

Application No. 09/514,245  
Atty. Doc. No. 065691/0176

**IN THE SPECIFICATION:**

Please amend the specification as follows:

On page 1, lines 5-7 of the Substitute Specification, make the following changes to the section titled "**Information on Related Applications**":

**--Information on Related Applications**

This application is a ~~continuation of U.S. Application No. 09/514,245, filed February 28, 2000, which is a 35 U.S.C. § 120~~ continuation-in-part of International Application No. PCT/FR98/02634, filed December 4, 1998.--.

**Amendments to the Claims:**

Claims 5-6, 20, and 22-24 are cancelled, without prejudice or disclaimer.

Claims 25-48 are being added.

This amendment adds, changes and/or deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, are presented. The text of all claims presently under examination is presented below in the listing of claims, and all claims are presented with an appropriate defined status identifier.

**Detailed and Complete Listing of Claims:**

1-24 (Cancelled).

25. (New) A vaccine comprising a nucleic acid having a nucleotide sequence with at least 90 % sequence identity to SEQ ID No. 25 and an acceptable pharmaceutical vehicle, wherein said nucleic acid encodes an immunogenic protein that induces a protective response effective against infection by a piglet weight loss disease circovirus.

26. (New) A vaccine according to claim 25, wherein said nucleotide sequence is SEQ ID No. 25.

27. (New) A vaccine according to claim 25, further comprising an adjuvant.

28. (New) A vaccine according to claim 25, wherein said nucleic acid has a nucleotide sequence with at least 95 % sequence identity to SEQ ID No. 25.

29. (New) A method of immunizing a mammal against piglet weight loss disease comprising administering to a mammal an effective amount of a vaccine,

wherein said vaccine comprises a nucleic acid having a nucleotide sequence with at least 90 % sequence identity to SEQ ID No. 25 and an acceptable pharmaceutical vehicle, wherein said nucleic acid encodes an immunogenic protein that induces a protective response effective against infection by a piglet weight loss disease circovirus.

30. (New) A method according to claim 29, wherein said nucleotide sequence is SEQ ID No. 25.

31. (New) A method according to claim 29, wherein said vaccine further comprises an adjuvant.

32. (New) A method according to claim 29, wherein said nucleic acid has a nucleotide sequence with at least 95 % sequence identity to SEQ ID No. 25.

33. (New) A vaccine comprising a nucleic acid having a nucleotide sequence with at least 95 % sequence identity to SEQ ID No. 25, an acceptable pharmaceutical vehicle, and an adjuvant, wherein said nucleic acid encodes an immunogenic protein that induces a protective response effective against infection by a piglet weight loss disease circovirus.

34. (New) A vaccine according to claim 33, wherein said nucleotide sequence is SEQ ID No. 25.

35. (New) A method of immunizing a mammal against piglet weight loss disease comprising administering to a mammal an effective amount of a vaccine according to claim 34.

36. (New) A method of immunizing a mammal against piglet weight loss disease comprising administering to a mammal an effective amount of a vaccine according to claim 33.

37. (New) A method for growing circoviruses, in particular porcine circoviruses (PCV), which comprises circoviruses obtained from an infected cell culture being, after one or more passages in cultures of porcine, bovine or human cells, developed in these cell cultures and a cytopathogenic effect occurring thereby.

38. (New) A method for neutralizing or removing circoviruses from biological material, which comprises treating it with an antibody-containing substrate such as porcine serum or human immunoglobulin or subjecting it to a pasteurization method.



39. (New) A method for detecting and quantifying antibodies directed against circoviruses by the ELISA method, which comprises circoviruses being incubated, after adsorption onto a support material, with the serum to be investigated and thus being bound to a primary antibody present in the serum, and subsequently a secondary, labeled antibody directed against the primary antibody being brought into contact therewith, and then the signal emitted by the bound, labeled antibody being measured.

40. (New) A method for detecting and quantifying the circovirus antigen by the ELISA method, which comprises an antibody against circoviruses which is bound to a support material being incubated with the serum to be investigated for circovirus antigen, and thus the antigen being bound, and the latter being brought into contact with a labeled antibody directed against the antigen and, after the unbound, labeled antibody has been washed out, the signal emitted by the bound, labeled antibody being measured.

41. (New) A vaccine, which comprises inactivated or avirulent circoviruses.

42. (New) A diagnostic aid which comprises inactivated or avirulent circoviruses.

43. (New) The use of circoviruses for investigating the capacity of a method for manufacturing pharmaceuticals of biological origin, of additives for the manufacture of pharmaceuticals or of a diagnostic aid to inactivate and/or remove circoviruses or related viruses.

44. (New) A method of growing a porcine circovirus (PCV), comprising culturing porcine cells that are infected with PWD circovirus type A (PCVA) and/or PWD circovirus of type B (PCVB).

45. (New) A method of neutralizing or removing a porcine circovirus (PCV) from a host, comprising administering to a host at least one antibody chosen from mono- and polyclonal antibodies, fragments of mono- and polyclonal antibodies, and chimeric antibodies, wherein said antibodies are capable of specifically recognizing a polypeptide expressed by porcine circovirus (PVC).

46. (New) A method for detecting and quantifying antibodies directed against circoviruses by the ELISA method, which comprises depositing a polypeptide expressed by a porcine circovirus (PCV) in the wells of a microtiter plate, introducing into said wells a biological sample containing PCV to be analyzed, incubating the microtiter plate, introducing into said wells of the microtiter plate labeled antibodies directed against pig immunoglobulins, the labeling of these antibodies having been carried out with the aid of an enzyme selected from those which are capable of hydrolyzing a substrate by modifying the absorption of the radiation of the substrate, at least at a determined wavelength, and detecting, by comparison with a control test, of the quantity of hydrolyzed substrate.

47. (New) A vaccine, which comprises an attenuated or inactivated viral particle comprising a nucleotide sequence coding for a polypeptide of PWD circovirus.

48. (New) A kit for diagnosing infection by a PWD circovirus, which comprises an attenuated or inactivated viral particle comprising a nucleotide sequence coding for a polypeptide of PWD circovirus.

**REMARKS**

**I. Disposition of the Claims**

Claims 25-48 are pending and new. Claims 5-6, 20, and 22-24 are cancelled, without prejudice or disclaimer, by this paper. Claims 1-4, 7-19, and 21 have already been cancelled.

New claims 25-36 belong with the elected group. New claims 37-48 embrace subject matter exceeding that of the elected group.

**II. Statement about the Amendment**

**A. Relation to Previously Allowed Claims**

Cancelled vaccine claims 5-6 and 22-24 were allowed. The PTO is invited to compare these claims with present claims 25-28.

Cancelled method claim 20 was allowed. The PTO is invited to compare this claim with present claims 29-31.

The PTO is invited to compare the previously allowed claims with present claims 32-36.

**B. Notice is hereby provided under 35 U.S.C. § 135(b).**

New claims 37-43 are copied verbatim from Application Publication No. 2002/0055189, (enclosed for considered) published May 9, 2002. Each claim's patentability is now being studied. Support for these claims has yet to be determined.

Along these lines, new claims 44-48 parallel those from Publication No. 2002/0055189. Support for each claim may be found, e.g., in the specification at the following passages:

**Claim 44:** Pages 52-*et seq.*; Example 1, especially page 53, lines 22-23.

**Claim 45:** Page 5, line 6; page 36, lines 18-20; page 42, l. 3; page 58, l. 15-*et seq.*, and Example 2.

**Claim 46:** Page 34, line 29-page 35, line 13, and Example 6.

**Claim 47:** Page 1, lines 10-11; page 42, line 13; and page 45, lines 11-15.

**Claim 48:** Page 1, lines 10-11; page 35, lines 14-17; page 42, line 13; and page 45, lines 11-15.

It is believed that claims of the '189 publication would correspond to count(s) based on pending claims 37-48 of this application. Applicants are contemplating preparing a request for interference under Rule 604 to be submitted at a later date.

As the present application is filed February 28, 2000, this amendment is not barred by 35 U.S.C. § 135(b).

### **III. Examiner's Interview**

Sean A. Passino (45,943) acknowledges and appreciates the Examiner's interview of May 5, 2003, initiated by the Examiner. During this interview, three major items were discussed: (1) claim for priority; (2) the continuity of disclosure of the parent application and the present substitute-specification, which is a CIP of International Application No. PCT/FR98/02634; and (3) the oath/declaration. Item (1) is addressed by the amendment to the specification correcting the previous amendment perfecting the claim for priority. Items (2)-(3) are addressed in subsequent sections.

### **IV. Continuity of Disclosure with PCT/FR98/02634**

The Examiner requested assistance determining the continuity of disclosure. In response, attached with this paper are Exhibits A-C. Exhibit A is a DeltaView comparison of Exhibits B and C. DeltaView is a commercial program that compares differences between the text of two files. Exhibit B, according to the undersigned's records, is the electronic copy of the English language translation of PCT/FR98/02634's publication (WO 99/29871), and Exhibit C, according to the undersigned's records, is an electronic copy of the present application as-filed. The comparison was performed using Exhibit B as the original document. Each Exhibit A-C is enclosed herewith.

The written description support for the pending claims is believed in the PCT/FR98/02634 as-filed. Citing the PCT's English language translation, Exhibit B, support may be found, e.g., in the following passages following each enumerated claim.

**Claim 25:** Page 1, line 15; page 10, line 35 (Seq. Id. No. 12); page 12, lines 20-22; and page 42, lines 22-25.

**Claim 26:** Page 40, lines 8-13.

**Claim 27:** Page 42, lines 22-25.

**Claim 28:** Page 12, lines 20-22.

**Claim 29:** Page 1, lines 15-16; page 10, line 35 (Seq. Id. No. 12); page 12, lines 20-22; page 42, lines 22-25; and page 45, lines 3-17.

**Claim 30:** Page 40, lines 8-13.

**Claim 31:** Page 42, lines 22-25.

**Claim 32:** Page 12, lines 20-22.

**Claim 33:** Page 1, line 15; page 10, line 35 (Seq. Id. No. 12); page 12, lines 20-22; and page 42, lines 22-25.

**Claim 34:** Page 40, lines 8-13.

**Claim 35:** Page 1, lines 15-16; page 10, line 35 (Seq. Id. No. 12); page 12, lines 20-22; page 42, lines 22-25; and page 45, lines 3-17.

**Claim 36:** Page 1, lines 15-16; page 10, line 35 (Seq. Id. No. 12); page 12, lines 20-22; page 42, lines 22-25; and page 45, lines 3-17.

## **V. Oath**

Even if the present application is a CIP of PCT/FR98/02634, the present oath filed June 26, 2002, is proper. According to Rule 63(b)(1), the oath must identify the application to which it is directed. In this case, the oath file June 26, 2002, is directed to the present application, i.e., U.S. Application No. 09/514,245. (Oath at page 1). Thus, the present oath is not defective, just because the present application is a CIP of PCT/FR98/02634.

## **VII. Conclusion**

In view of the foregoing amendments and remarks, Applicants respectfully request reconsideration and reexamination of this application and the timely allowance of the pending claims.

Application No. 09/514,245  
Atty. Doc. No. 065691/0176


In the event the Examiner has any questions, the Examiner is invited to telephone the undersigned representative at the number indicated below.

Respectfully submitted,

Date

6/4/3

By

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FOR

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Should additional fees be necessary in connection with the filing of this paper, or if a petition for extension of time is required for timely acceptance of same, the Commissioner is hereby authorized to charge Deposit Account No. 19-0741 for any such fees; and applicant(s) hereby petition for any needed extension of time.

Enclosures:

Exhibits A-C

U.S. Application Publication No. 2002/0055189



WO 99/29871\*

-1-Attorney Docket No. 65691/176

PCT/FR98/02634

**Title of the Invention**CIRCOVIRUS SEQUENCES ASSOCIATED WITH PIGLET  
WEIGHT LOSS DISEASE (PWD)RECEIVED  
JUN 06 2003  
TECH CENTER 1600/29005 **Information on Related Applications**

The present application claims the priority benefit, under 35 U.S.C. § 119, of International Application No. PCT/FR98/02634, filed December 4, 1998.

**Background of the Invention**

10 The invention relates to the genomic sequence and nucleotide sequences coding for polypeptides of PWD circovirus, such as the structural and nonstructural polypeptides of said circovirus, as well as vectors including said sequences and cells or animals transformed by these vectors. The invention likewise relates to methods for detecting these nucleic acids or polypeptides and kits for  
15 diagnosing infection by the PWD circovirus. The invention is also directed ~~to~~ a method for selecting compounds capable of modulating the viral infection. The invention ~~finally~~further comprises pharmaceutical compositions, ~~especially~~including vaccines, for the prevention and/or the treatment of viral infections by PWD circovirus as well as the use of a vector according to the  
20 invention for the prevention and/or the treatment of diseases by gene therapy.

Piglet weight loss disease (PWD)—~~or~~ alternatively called fatal piglet wasting (FPW) has been widely described in North America (Harding, J.C., 1997), and authors have reported the existence of a relationship between this pathology and the presence of porcine circovirus (Daft, B. et al., 1996; Clark, E.G., 1997;  
25 Harding, J.C., 1997; Harding, J.C. and Clark, E.G., 1997; Nayar, G.P. et al., 1997). A porcine circovirus has already been demonstrated in established lines of cell cultures derived from pigs and chronically infected (Tischer, I., 1986, 1988, 1995; Dulac, G.C., 1989; Edwards, S., 1994; Allan, G.M., 1995 and McNeilly, F., 1996). This virus, during experimental infection of piglets, does not prove



pathogenic for pigs (Tischer, I., 1986, Horner, G.W., 1991) and its nucleotide sequence has been determined and characterized (Tischer, I., 1982; Meehan, B.M. et al., 1997; Mankertz., A., 1997). The porcine circovirus, called PCV virus, is part of the circovirus genus of the circoviridae family (Murphy, F.A. et al., 1995) whose virion has a circular DNA of size between 1.7 and 2.3 kb, which DNA comprises three open reading frames (ORF1 to ORF3), coding for a replication protein REP involved in the initiation and termination phase of rolling circular replication (RCR) (Heyraud-Nitschke, F., et al., 1995; Harding, M.R. et al., 1993; Hanson, S.F. et al., 1995; Fontes, E.P.B. et al., 1994), coding for a capsid protein (Boulton, L.H. et al., 1997; Hackland, A.F. et al., 1994; Chu, P.W.G. et al., 1993) and coding for a nonstructural protein called a dissemination protein (Lazarowitz., S.G. et al., 1989).

The authors of the present invention have noticed that the clinical signs perceptible in pigs and linked to infection by the PWD circovirus are very distinctive. These manifestations in general appear in pigs of 8 to 12 weeks of age, weaned for 4 to 8 weeks. The first signs are hypotonia without it being possible to speak of prostration. Rapidly (48 hours), the flanks hollow, the line of the spine becomes apparent, and the pigs "blanch". These signs are in general accompanied by hyperthermia, anorexia and most often by respiratory signs (coughing, dyspnea, polypnea). Transitory diarrhea can likewise appear. The disease state phase lasts approximately one month at the end of which the rate of mortality varies from 5 to 20%. To these mortalities, it is expedient to add a variable proportion (5-10%) of cadaveric animals which are no longer able to present an economic future. It is to be noted that outside of this critical stage of the end of post-weaning, no anomaly appears on the farms. In particular, the reproductive function is totally maintained.

On the epidemiological level, the first signs of this pathology appeared at the start of 1995 in the east of the Côtes d'Armor ~~department~~region in France, and the farms affected are especially confined to this area of the ~~department~~region. In December 1996, the number of farms concerned could not be evaluated with



precision because of the absence of a specific laboratory diagnostic method or of an epidemiological surveillance system of the livestock. Based on the clinical facts as well as on results of postmortem examinations supplied by veterinarians, it is possible to estimate this number as several dozen (80-100). The contagiousness of the disease is weak to moderate. Cases are being reported outside the initial area and for the majority are following the transfer of animals coming from farms familiar with the problem. On the other hand, a characteristic of the condition is its strong remanence. Thus, farms which have been affected for a year are still affected in spite of the massive administration of therapeutics. Farms with clinical expression are drawn from various categories of specialization (breeders/fatteners, post-weaners/ fatteners) and different economic structures are concerned. In addition, the disorders appear even in farms where the rules of animal husbandry are respected.

Numerous postmortem examinations have been carried out either on farms or in the laboratory. The elements of the lesional table are disparate. The most constant macroscopic lesions are pneumonia which sometimes appears in patchy form as well as hypertrophy of the lymphatic ganglia. The other lesions above all affect the thoracic viscera including, especially, pericarditis and pleurisy. However, arthritis and gastric ulcers are also observed. The lesions revealed in the histological examination are essentially situated at the pulmonary level (interstitial pneumonia), ganglionic level (lymphoid depletion of the lymph nodes, giant cells) and renal level (glomerulonephritis, vasculitis). The infectious agents have been the subject of wide research. It has been possible to exclude the intervention of pestiviruses and Aujeszky's disease. The disorders appear in the seropositive PDRS (Porcine Dysgenic and Respiratory Syndrome, an infection linked to an arteriovirus) herds, but it has not been possible to establish the role of the latter in the genesis of the disorders (the majority of the farms in Brittany are PDRS seropositive).

The authors of the present invention, with the aim of identifying the etiological agent responsible for PWD, have carried out "contact" tests between piglets which are obviously "ill" and SPF pigs (specific pathogen-free) from

CNEVA (Centre National d'Etudes Vétérinaires et Alimentaires, France). These tests allow the development of signs comparable to those observed on the farm to be observed in protected animal houses. The discrete signs such as moderate hyperthermia, anorexia and intermittent diarrhea appeared after one week of contact. It must be noted that the PDRS virus only diffused subsequent to the clinical signs. In addition, inoculations of organ homogenates of sick animals to healthy pigs allowed signs related to those observed on the farms to be reproduced, although with a lower incidence, linked to the favorable conditions of upkeep of the animals in the experimental installations.

Thus, the authors of the present invention have been able to demonstrate that the pathological signs appear as a well-defined entity affecting the pig at a particular stage of its growth.

This pathology has never been described in France. However, sparse information, especially Canadian, relates to similar facts.

The disorders cannot be mastered with the existing therapeutics.

The data collected both on the farm and by experimentation have allowed the following points to be highlighted:

- PWD is transmissible but its contagiousness is not very high,
- its etiological origin is of infectious and probably viral nature,
- PWD has a persistent character in the affected farms.

Considerable economic consequences ensue for the farms.

Thus, there is currently a significant need for a specific and sensitive diagnostic, whose production is practical and rapid, allowing the early detection of the infection.

A reliable, sensitive and practical test which allows the distinction between strains of porcine circovirus (PCV) is thus strongly desirable.

On the other hand, a need for efficient and well-tolerated treatment of infections with PWD circovirus likewise remains desirable, no vaccine currently being available against PWD circovirus.

Concerning PWD circovirus, it will probably be necessary to understand the role of the immune defense in the physiology and the pathology of the disease to develop satisfactory vaccines.

Fuller information concerning the biology of these strains, their interactions with their hosts, the associated infectivity phenomena and those of escape from the immune defenses of the host especially, and finally their implication in the development of associated pathologies, will allow a better understanding of these mechanisms. Taking into account the facts which have been mentioned above and which show in particular the limitations of combatting infection by the PWD circovirus, it is thus essential today on the one hand to develop molecular tools, especially starting from a better genetic knowledge of the PWD circovirus, ~~but~~ and likewise to perfect novel preventive and therapeutic treatments, novel methods of diagnosis and specific, efficacious and tolerated novel vaccine strategies. This is precisely the subject of the present invention.

### Summary of the Invention

The present invention relates to vaccines comprising a nucleotide sequence of the genome of Porcine circovirus type B, or a homologue or fragment thereof, and an acceptable pharmaceutical or veterinary vehicle. In one embodiment of the invention, the nucleotide sequence is selected from SEQ ID No. 15, SEQ ID No. 19, SEQ ID No. 23, or SEQ ID No. 25, or a homologue or fragment thereof. In another embodiment of the invention, the homologue has at least 80% sequence identity to SEQ ID No. 15, SEQ ID No. 19, SEQ ID No. 23 or SEQ ID No. 25. In yet another embodiment, the vaccines further comprising an adjuvant

The present invention also relates to vaccines comprising a polypeptide encoded by a nucleotide sequence of the genome of PCVB, or a homologue or fragment thereof, and an acceptable pharmaceutical or veterinary vehicle. In one embodiment, the homologue has at least 80% sequence identity to SEQ ID No. 15, SEQ ID No. 19, SEQ ID No. 23 or SEQ ID No. 25. In another embodiment of the

invention, the nucleotide sequence is selected from SEQ ID No. 23 or SEQ ID No. 25, or a homologue or fragment thereof. In still another embodiment, the polypeptide has the amino acid sequence of SEQ ID No. 24 or SEQ ID No. 26. In yet another embodiment, the homologue has at least 80% sequence identity to SEQ ID No. 24 or SEQ ID No. 26. In another embodiment, the polypeptide has the amino acid sequence of SEQ ID No. 29, SEQ ID No. 30, SEQ ID No. 31, or SEQ ID No. 32.

A further aspect of the invention relates to vaccines comprising a vector and an acceptable pharmaceutical or veterinary vehicle, the vector comprising a nucleotide sequence of the genome of Porcine circovirus type B, or a homologue or fragment thereof. In one embodiment, the vaccine further comprises a gene coding for an expression product capable of inhibiting or retarding the establishment or development of a genetic or acquired disease.

The present invention also relates to vaccines comprising a cell and an acceptable pharmaceutical or veterinary vehicle, wherein the cell is transformed with a nucleotide sequence of the genome of Porcine circovirus type B, or a homologue or fragment thereof.

Still further, the present invention relates to vaccines comprising a pharmaceutically acceptable vehicle and a single polypeptide, wherein the single polypeptide consists of SEQ ID No. 26.

Additionally, the present invention relates to methods of immunizing a mammal against piglet weight loss disease comprising administering to a mammal an effective amount of the vaccines described above.

These and other aspects of the invention will become apparent to the skilled artisan in view of the teachings contained herein.

**Brief Description of the Drawings**

Figure 1: Experimental scheme which has made it possible to bring about the isolation and the identification of the circovirus associated with PWD of type A and B.

5           Test 1: experimental reproduction of the PWD by inoculation of pig organ homogenates from farms affected by PWD.

Test 2: experimental reproduction of PWD.

Test 3: experimental reproduction of PWD.

Test 4: no experimental reproduction of PWD.

10

Figure 2: Organization of the genome of the circovirus associated with PWD of type A (PCVA)

- strand of (+) polarity (SEQ ID No. 1);

15           - strand of (-) polarity (SEQ ID No. 5, represented according to the orientation 3' → 5');

- sequences of amino acids of proteins encoded by the two DNA strands in the three possible reading frames SEQ ID NOS: 2-4 and 6-8 respectively.

20           Figure 3: Alignment of the nucleotide sequence SEQ ID No. 1 of the PWD circovirus of type A (PCVA) and of the MEEHAN SEQ ID No. 163 strain and MANKERTZ SEQ ID No. 164 strain circoviruses of the porcine cell lines.

25           Figure 4: Alignment of the sequence of amino acids SEQ ID No. 10 of a polypeptide encoded by the nucleotide sequence SEQ ID No. 9 (ORF1) of the PWD circovirus of type A (PCVA) and of corresponding nucleotide sequences of the MEEHAN SEQ ID No. 165 strain and MANKERTZ SEQ ID No. 166 strain circoviruses of the porcine cell lines.

5 Figure 5: Alignment of the sequence of amino acids SEQ ID No. 12 of a polypeptide encoded by the nucleotide sequence SEQ ID No. 11 (ORF2) of the PWD circovirus of type A (PCVA) and of corresponding nucleotide sequences of the MEEHAN SEQ ID No. 167 strain and MANKERTZ SEQ ID No. 168 strain circoviruses of the porcine cell lines.

10 Figure 6: Alignment of the sequence of amino acids SEQ ID No. 14 of a polypeptide encoded by the nucleotide sequence SEQ ID No. 13 (ORF3) of the PWD circovirus of type A (PCVA) and of corresponding nucleotide sequences of the MEEHAN SEQ ID No. 169 strain and MANKERTZ SEQ ID No. 170 strain circoviruses of the porcine cell lines.

Figure 7: Western blot analysis of recombinant proteins of the PWD circovirus of type A (PCVA).

15 The analyses were carried out on cell extracts of Sf9 cells obtained after infection with recombinant baculovirus PCF ORF 1.

Figure 8: Organization of the genome of the circovirus associated with the PWD of type B (PCVB)

20 - strand of (+) polarity (SEQ ID No. 15);  
- strand of (-) polarity (SEQ ID No. 19, represented according to the orientation 3' → 5');  
- sequence of amino acids of proteins encoded by the two DNA strands in the three possible reading frames SEQ ID NOS: 16-18 and 20-22 respectively.

25

Figure 9: Evolution of the daily mean gain (DMG) of pig farms affected by piglet weight loss disease (PWD), placed under experimental conditions.



Figure 10: DMG compared for the 3 batches of pigs (F1, F3 and F4) calculated over a period of 28 days, after vaccination test.

5 Figure 11: Hyperthermia greater than 41°C, expressed as a percentage compared for the 3 batches of pigs (F1, F3 and F4) calculated per week over a period of 28 days, after vaccination test.

10 Figure 12: Membranes of peptide spots corresponding to the ORF2s revealed with the aid of an infected pig serum, originating from a conventional farm.

The numbers of specific peptides of the circovirus of type B as well as their nonreactive homologs (type A) are indicated in bold.

The nonspecific immunogenic peptides are indicated in italics.

15 Figure 13: Alignment of amino acid sequences of proteins encoded by the ORF2 of the PWD circovirus of type A SEQ ID No. 12 and by the ORF'2 of the PWD circovirus of type B SEQ ID No. 26. The position of 4 peptides corresponding to specific epitopes of the PWD circovirus of type B is indicated on the corresponding sequence by a bold line, their homolog on the sequence of the  
20 PWD circovirus of type A is likewise indicated by an ordinary line.

Figure 14: Charts the results of experiments that demonstrate, in terms of percent hyperthermia, that vaccination with ORF'1 and ORF'2 of PCV-B enhances the level of protection in swine challenged with PCV-B.

25

Figure 15: Charts the results of experiments that demonstrate, in terms of animal growth, that vaccination with ORF'1 and ORF'2 of PCV-B enhances the level of protection in swine challenged with PCV-B.

Figure 16: Immunoperoxidase staining of PK15 cells at 24 h post-transfection with the pcDNA3/ORF'2 plasmid. Expression of PCVB ORF'2 was confirmed by IPMA following incubation in the presence of the swine anti-PCVB monospecific serum

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### **Detailed Description of the Invention**

The present invention relates to nucleotide sequences of the genome of PWD circovirus selected from the sequences SEQ ID No. 1, SEQ ID No. 275, SEQ ID No. 9715, SEQ ID No. 1019 or one of their fragments.

10

The nucleotide sequences of sequences SEQ ID No. 1 and SEQ ID No. 25 correspond respectively to the genome sequence of the strand of (+) polarity and of the strand of (-) polarity of the PWD circovirus of type A (or PCVA), the sequence SEQ ID No. 25 being represented according to the orientation 5'→3'.

15

The nucleotide sequences of sequences SEQ ID No. 915 and SEQ ID No. 1019 correspond respectively to the genome sequence of the strand of (+) polarity and of the strand of (-) polarity of the PWD circovirus of type B (or PCVB), the sequence SEQ ID No. 1019 being represented according to the orientation 5'→3'.

20

The present invention likewise relates to nucleotide sequences, characterized in that they are selected from:

25

- a) a nucleotide sequence of a specific fragment of the sequence SEQ ID No. 1, SEQ ID No. 275, SEQ ID No. 9715, SEQ ID No. 1019 or one of their fragments;
- b) a nucleotide sequence homologous to a nucleotide sequence such as defined in a);
- c) a nucleotide sequence complementary to a nucleotide sequence such as defined in a) or b), and a nucleotide sequence of their corresponding RNA;
- d) a nucleotide sequence capable of hybridizing under stringent conditions with a sequence such as defined in a), b) or c);



- e) a nucleotide sequence comprising a sequence such as defined in a), b), c) or d); and
- f) a nucleotide sequence modified by a nucleotide sequence such as defined in a), b), c), d) or e).

5 Nucleotide, polynucleotide or nucleic acid sequence will be understood according to the present invention as meaning both a double-stranded or single-stranded DNA in the monomeric and dimeric (so-called in tandem) forms and the transcription products of said DNAs.

10 It must be understood that the present invention does not relate to the genomic nucleotide sequences taken in their natural environment, that is to say in the natural state. It concerns sequences which it has been possible to isolate, purify or partially purify, starting from separation methods such as, for example, ion-exchange chromatography, by exclusion based on molecular size, or by affinity, or  
15 alternatively fractionation techniques based on solubility in different solvents, or starting from methods of genetic engineering such as amplification, cloning and subcloning, it being possible for the sequences of the invention to be carried by vectors.

The nucleotide sequences SEQ ID No. 1 and SEQ ID No. ~~9~~15 were obtained by sequencing of the genome by the Sanger method.

20 Nucleotide sequence fragment according to the invention will be understood as designating any nucleotide fragment of the PWD circovirus, type A or B, of length of at least 8 nucleotides, preferably at least 12 nucleotides, and even more preferentially at least 20 consecutive nucleotides of the sequence from which it originates.

25 Specific fragment of a nucleotide sequence according to the invention will be understood as designating any nucleotide fragment of the PWD circovirus, type A or B, having, after alignment and comparison with the corresponding fragments of known porcine circoviruses, at least one nucleotide or base of different nature. For example, the specific nucleotide fragments of the PWD circovirus of type A can

easily be determined by referring to Figure 3 of the present invention in which the nucleotides or bases of the sequence SEQ ID No. 1 (circopordfp) are shown which are of different nature, after alignment of said sequence SEQ ID No. 1 with the other two sequences of known porcine circovirus (circopormeeh and circopormank).

5 Homologous nucleotide sequence in the sense of the present invention is understood as meaning a nucleotide sequence having at least a percentage identity with the bases of a nucleotide sequence according to the invention of at least 80%, preferably 90% or 95%, this percentage being purely statistical and it being possible to distribute the differences between the two nucleotide sequences at random and  
10 over the whole of their length.

Specific homologous nucleotide sequence in the sense of the present invention is understood as meaning a homologous nucleotide sequence having at least one nucleotide sequence of a specific fragment, such as defined above. Said "specific" homologous sequences can comprise, for example, the sequences  
15 corresponding to the genomic sequence or to the sequences of its fragments representative of variants of PWD circovirus of type A or B. These specific homologous sequences can thus correspond to variations linked to mutations within strains of PWD circovirus of type A and B, and especially correspond to truncations, substitutions, deletions and/or additions of at least one nucleotide. Said  
20 homologous sequences can likewise correspond to variations linked to the degeneracy of the genetic code.

The term "degree or percentage of sequence homology" refers to "degree or percentage of sequence identity between two sequences after optimal alignment" as defined in the present application.

25 Two amino-acids or nucleotidic sequences are said to be "identical" if the sequence of amino-acids or nucleotidic residues, in the two sequences is the same when aligned for maximum correspondence as described below. Sequence comparisons between two (or more) peptides or polynucleotides are typically performed by comparing sequences of two optimally aligned sequences over a

segment or "comparison window" to identify and compare local regions of sequence similarity. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman, *Ad. App. Math* 2: 482 (1981), by the homology alignment algorithm of Neddleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. (U.S.A.)* 85: 2444 (1988), by computerized implementation of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by visual inspection.

"Percentage of sequence identity" (or degree or identity) is determined by comparing two optimally aligned sequences over a comparison window, where the portion of the peptide or polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) **for optimal alignment** of the two sequences. The percentage is calculated by determining the number of positions at which the identical amino-acid residue or nucleic acid base occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

The definition of sequence identity given above is the definition that would use one of skill in the art. The definition by itself does not need the help of any algorithm, said algorithms being helpful only to achieve the optimal alignments of sequences, rather than the calculation of sequence identity.

From the definition given above, it follows that there is a well defined and only one value for the sequence identity between two compared sequences which value corresponds to the value obtained for the best or optimal alignment.

In the BLAST N or BLAST P "BLAST 2 sequence", software which is available in the web site <http://www.ncbi.nlm.nih.gov/gorf/bl2.html>, and habitually used by the inventors and in general by the skilled man for comparing and

determining the identity between two sequences, gap cost which depends on the sequence length to be compared is directly selected by the software (i.e. 11.2 for substitution matrix BLOSUM-62 for length > 85).

5 In the present description, PWD circovirus will be understood as designating the circoviruses associated with piglet weight loss disease (PWD) of type A (PCVA) or type B (PCVB), defined below by their genomic sequence, as well as the circoviruses whose nucleic sequences are homologous to the sequences of PWD circoviruses of type A or B, such as in particular the circoviruses corresponding to variants of the type A or of the type B.

10 Complementary nucleotide sequence of a sequence of the invention is understood as meaning any DNA whose nucleotides are complementary to those of the sequence of the invention, and whose orientation is reversed (antiparallel sequence).

15 Hybridization under conditions of stringency with a nucleotide sequence according to the invention is understood as meaning a hybridization under conditions of temperature and ionic strength chosen in such a way that they allow the maintenance of the hybridization between two fragments of complementary DNA.

20 By way of illustration, conditions of great stringency of the hybridization step with the aim of defining the nucleotide fragments described above are advantageously the following.

The hybridization is carried out at a preferential temperature of 65°C in the presence of SSC buffer, 1 × SSC corresponding to 0.15 M NaCl and 0.05 M Na citrate. The washing steps, for example, can be the following:

25 - 2 × SSC, at ambient temperature followed by two washes with 2 × SSC, 0.5% SDS at 65°C; 2 × 0.5 × SSC, 0.5% SDS; at 65°C for 10 minutes each.

The conditions of intermediate stringency, using, for example, a temperature of 42°C in the presence of a 2 × SSC buffer, or of less stringency, for

example a temperature of 37°C in the presence of a 2 × SSC buffer, respectively require a globally less significant complementarity for the hybridization between the two sequences.

5 The stringent hybridization conditions described above for a polynucleotide with a size of approximately 350 bases will be adapted by the person skilled in the art for oligonucleotides of greater or smaller size, according to the teaching of Sambrook et al., 1989.

10 Among the nucleotide sequences according to the invention, those are likewise preferred which can be used as a primer or probe in methods allowing the homologous sequences according to the invention to be obtained, these methods, such as the polymerase chain reaction (PCR), nucleic acid cloning and sequencing, being well known to the person skilled in the art.

15 Among said nucleotide sequences according to the invention, those are again preferred which can be used as a primer or probe in methods allowing the presence of PWD circovirus or one of its variants such as defined below to be diagnosed.

20 The nucleotide sequences according to the invention capable of modulating, of inhibiting or of inducing the expression of PWD circovirus gene, and/or capable of modulating the replication cycle of PWD circovirus in the host cell and/or organism are likewise preferred. Replication cycle will be understood as designating the invasion and the multiplication of PWD circovirus, and its propagation from host cell to host cell in the host organism.

25 Among said nucleotide sequences according to the invention, those corresponding to open reading frames, called ORF sequences, and coding for polypeptides, such as, for example, the sequences SEQ ID No. 39 (ORF1), SEQ ID No. 411 (ORF2) and SEQ ID No. 513 (ORF3) respectively corresponding to the nucleotide sequences between the positions 47 and 985 determined with respect to the position of the nucleotides on the sequence SEQ ID No. 1, the positions 1723 and 1022 and the positions 658 and 38 with respect to the position of the nucleotides on the sequence SEQ ID No. 25 (represented according to the orientation 3'→5'),

the ends being included, or alternatively the sequences SEQ ID No. 1123 (ORF'1),  
SEQ ID No. 1225 (ORF'2) and SEQ ID No. 1327 (ORF'3), respectively  
corresponding to the sequences between the positions 51 and 995 determined with  
respect to the position of the nucleotides on the sequence SEQ ID No. 915, the  
5 positions 1734 and 1033 and the positions 670 and 357, the positions being  
determined with respect to the position of the nucleotides on the sequence SEQ ID  
No. 1019 (represented according to the orientation 3'→5'), the ends being  
included, are finally preferred.

The nucleotide sequence fragments according to the invention can be  
10 obtained, for example, by specific amplification, such as PCR, or after digestion  
with appropriate restriction enzymes of nucleotide sequences according to the  
invention, these methods in particular being described in the work of Sambrook et  
al., 1989. Said representative fragments can likewise be obtained by chemical  
15 synthesis when their size is not very large and according to methods well known to  
persons skilled in the art.

Modified nucleotide sequence will be understood as meaning any nucleotide  
sequence obtained by mutagenesis according to techniques well known to the person  
skilled in the art, and containing modifications with respect to the normal sequences  
according to the invention, for example mutations in the regulatory and/or promoter  
20 sequences of polypeptide expression, especially leading to a modification of the rate  
of expression of said polypeptide or to a modulation of the replicative cycle.

Modified nucleotide sequence will likewise be understood as meaning any  
nucleotide sequence coding for a modified polypeptide such as defined below.

The present invention relates to nucleotide sequences of PWD circovirus  
25 according to the invention, characterized in that they are selected from the  
sequences SEQ ID No. 3, ~~SEQ ID No. 4~~, ~~SEQ ID No. 5~~, 9, SEQ ID No.  
11, SEQ ID No. 1213, SEQ ID No. 1323, SEQ ID No. 25, SEQ ID No. 27 or  
one of their fragments.



The invention likewise relates to nucleotide sequences characterized in that they comprise a nucleotide sequence selected from:

a) a nucleotide sequence SEQ ID No. ~~3~~, ~~SEQ ID No. 4~~, ~~SEQ ID No. 5~~, 9, SEQ ID No. 11, SEQ ID No. ~~12~~, 13, SEQ ID No. ~~13~~23, SEQ ID No. 25, SEQ ID No. 27 or one of their fragments;

b) a nucleotide sequence of a specific fragment of a sequence such as defined in a);

c) a homologous nucleotide sequence having at least 80% identity with a sequence such as defined in a) or b);

d) a complementary nucleotide sequence or sequence of RNA corresponding to a sequence such as defined in a), b) or c); and

e) a nucleotide sequence modified by a sequence such as defined in a), b), c) or d).

As far as homology with the nucleotide sequences SEQ ID No. ~~3~~, ~~SEQ ID No. 4~~, ~~SEQ ID No. 5~~, 9, SEQ ID No. 11, SEQ ID No. ~~12~~, 13, SEQ ID No. ~~13~~23, SEQ ID No. 25, SEQ ID No. 27 or one of their fragments is concerned, the homologous, especially specific, sequences having a percentage identity with one of the sequences SEQ ID No. ~~3~~, ~~SEQ ID No. 4~~, ~~SEQ ID No. 5~~, 9, SEQ ID No. 11, SEQ ID No. ~~12~~, 13, SEQ ID No. ~~13~~23, SEQ ID No. 25, SEQ ID No. 27 or one of their fragments of at least 80%, preferably 90% or 95%, are preferred. Said specific homologous sequences can comprise, for example, the sequences corresponding to the sequences ORF1, ORF2, ORF3, ORF'1, ORF'2 and ORF'3 of PWD circovirus variants of type A or of type B. In the same manner, these specific homologous sequences can correspond to variations linked to mutations within strains of PWD circovirus of type A or of type B and especially correspond to truncations, substitutions, deletions and/or additions of at least one nucleotide.

Among nucleotide sequences according to the invention, the sequence SEQ ID No. ~~11~~23 which has a homology having more than 80% identity with the

sequence SEQ ID No. ~~3~~9, as well as the sequence SEQ ID No. ~~12~~25, are especially preferred.

Preferably, the invention relates to the nucleotide sequences according to the invention, characterized in that they comprise a nucleotide sequence  
5 selected from the following sequences:

- a) SEQ ID No. 33 170 5' TGTGGCGA 3';
- b) SEQ ID No. 34 450 5' AGTTTCCT 3';
- c) SEQ ID No. 35 1026 5' TCATTTAGAGGGTCTTTCAG 3';
- d) SEQ ID No. 36 1074 5' GTCAACCT 3';
- 10 e) SEQ ID No. 37 1101 5' GTGGTTGC 3';
- f) SEQ ID No. 38 1123 5' AGCCCAGG 3';
- g) SEQ ID No. 39 1192 5' TTGGCTGG 3';
- h) SEQ ID No. 40 1218 5' TCTAGCTCTGGT 3';
- i) SEQ ID No. 41 1501 5' ATCTCAGCTCGT 3';
- 15 j) SEQ ID No. 42 1536 5' TGTCCTCCTCTT 3';
- k) SEQ ID No. 43 1563 5' TCTCTAGA 3';
- l) SEQ ID No. 44 1623 5' TGTACCAA 3';
- m) SEQ ID No. 45 1686 5' TCCGTCTT 3';

and their complementary sequences.

20 In the list of nucleotide sequences a)-m) above, the underlined nucleotides are mutated with respect to the two known sequences of circovirus which are nonpathogenic to pigs. The number preceding the nucleotide sequence represents the position of the first nucleotide of said sequence in the sequence SEQ ID No. 1.

25 The invention comprises the polypeptides encoded by a nucleotide sequence according to the invention, preferably a polypeptide whose sequence is represented by a fragment, especially a specific fragment, of one of the six sequences of amino acids represented in Figure 2, these six amino acid sequences corresponding to the polypeptides which can be encoded according to one of the three possible reading



frames of the sequence SEQ ID No. 1 or of the sequence SEQ ID No. ~~2~~5, or a polypeptide whose sequence is represented by a fragment, especially a specific fragment, of one of the six sequences of amino acids shown in Figure 8, these six sequences of amino acids corresponding to the polypeptides which can be encoded according to one of the three possible reading frames of the sequence SEQ ID No. ~~9~~15 or of the sequence SEQ ID No. ~~10~~19.

The invention likewise relates to the polypeptides, characterized in that they comprise a polypeptide selected from the amino acid sequences SEQ ID No. ~~6~~10, SEQ ID No. ~~7~~12, SEQ ID No. 14, SEQ ID No. ~~15~~24, SEQ ID No. ~~16~~26, SEQ ID No. 28 or one of their fragments.

Among the polypeptides according to the invention, the polypeptide of amino acid sequence SEQ ID No. ~~14~~24 which has a homology having more than 80% identity with the sequence SEQ ID No. ~~6~~10, as well as the polypeptide of sequence SEQ ID No. ~~15~~26, are especially preferred.

The invention also relates to the polypeptides, characterized in that they comprise a polypeptide selected from:

- a) a specific fragment of at least 5 amino acids of a polypeptide of an amino acid sequence according to the invention;
- b) a polypeptide homologous to a polypeptide such as defined in a);
- 20 c) a specific biologically active fragment of a polypeptide such as defined in a) or b); and
- d) a polypeptide modified by a polypeptide such as defined in a), b) or c).

Among the polypeptides according to the invention, the polypeptides of amino acid sequences SEQ ID No. ~~17~~29, SEQ ID No. ~~18~~30, SEQ ID No. ~~19~~31 and SEQ ID No. ~~20~~32 are also preferred, these polypeptides being especially capable of specifically recognizing the antibodies produced during infection by the PWD circovirus of type B. These polypeptides thus have epitopes specific for the PWD circovirus of type B and can thus be used in particular in the diagnostic field

or as immunogenic agent to confer protection in pigs against infection by PWD circovirus, especially of type B.

In the present description, the terms polypeptide, peptide and protein are interchangeable.

5           It must be understood that the invention does not relate to the polypeptides in natural form, that is to say that they are not taken in their natural environment but that they can be isolated or obtained by purification from natural sources, or else obtained by genetic recombination, or alternatively by chemical synthesis and that they can thus contain unnatural amino acids, as will be described below.

10           Polypeptide fragment according to the invention is understood as designating a polypeptide containing at least 5 consecutive amino acids, preferably 10 consecutive amino acids or 15 consecutive amino acids.

15           In the present invention, specific polypeptide fragment is understood as designating the consecutive polypeptide fragment encoded by a specific fragment nucleotide sequence according to the invention.

20           Homologous polypeptide will be understood as designating the polypeptides having, with respect to the natural polypeptide, certain modifications such as, in particular, a deletion, addition or substitution of at least one amino acid, a truncation, a prolongation, a chimeric fusion, and/or a mutation. Among the homologous polypeptides, those are preferred whose amino acid sequence has at least 80%, preferably 90%, homology with the sequences of amino acids of polypeptides according to the invention.

25           Specific homologous polypeptide will be understood as designating the homologous polypeptides such as defined above and having a specific fragment of polypeptide according to the invention.

          In the case of a substitution, one or more consecutive or nonconsecutive amino acids are replaced by "equivalent" amino acids. The expression "equivalent" amino acid is directed here at designating any amino acid capable of being substituted by one of the amino acids of the base structure without, however,

essentially modifying the biological activities of the corresponding peptides and such that they will be defined by the following.

5        These equivalent amino acids can be determined either by depending on their structural homology with the amino acids which they substitute, or on results of comparative tests of biological activity between the different polypeptides, which are capable of being carried out.

10       By way of example, the possibilities of substitutions capable of being carried out without resulting in an extensive modification of the biological activity of the corresponding modified polypeptides will be mentioned, the replacement, for example, of leucine by valine or isoleucine, of aspartic acid by glutamic acid, of glutamine by asparagine, of arginine by lysine etc., the reverse substitutions naturally being envisageable under the same conditions.

15       The specific homologous polypeptides likewise correspond to polypeptides encoded by the specific homologous nucleotide sequences such as defined above and thus comprise in the present definition the polypeptides which are mutated or correspond to variants which can exist in PWD circovirus, and which especially correspond to truncations, substitutions, deletions and/or additions of at least one amino acid residue.

20       Specific biologically active fragment of a polypeptide according to the invention will be understood in particular as designating a specific polypeptide fragment, such as defined above, having at least one of the characteristics of polypeptides according to the invention, especially in that it is:

- capable of inducing an immunogenic reaction directed against a PWD circovirus; and/or
- 25       -        capable of being recognized by a specific antibody of a polypeptide according to the invention; and/or
- capable of linking to a polypeptide or to a nucleotide sequence of PWD circovirus; and/or

- capable of exerting a physiological activity, even partial, such as, for example, a dissemination or structural (capsid) activity; and/or
- capable of modulating, of inducing or of inhibiting the expression of PWD circovirus gene or one of its variants, and/or capable of modulating the replication cycle of PWD circovirus in the cell and/or the host organism.

The polypeptide fragments according to the invention can correspond to isolated or purified fragments naturally present in a PWD circovirus or correspond to fragments which can be obtained by cleavage of said polypeptide by a proteolytic enzyme, such as trypsin or chymotrypsin or collagenase, or by a chemical reagent, such as cyanogen bromide (CNBr) or alternatively by placing said polypeptide in a very acidic environment, for example at pH 2.5. Such polypeptide fragments can likewise just as easily be prepared by chemical synthesis, from hosts transformed by an expression vector according to the invention containing a nucleic acid allowing the expression of said fragments, placed under the control of appropriate regulation and/or expression elements.

"Modified polypeptide" of a polypeptide according to the invention is understood as designating a polypeptide obtained by genetic recombination or by chemical synthesis as will be described below, having at least one modification with respect to the normal sequence. These modifications will especially be able to bear on amino acids at the origin of a specificity, of pathogenicity and/or of virulence, or at the origin of the structural conformation, and of the capacity of membrane insertion of the polypeptide according to the invention. It will thus be possible to create polypeptides of equivalent, increased or decreased activity, and of equivalent, narrower, or wider specificity. Among the modified polypeptides, it is necessary to mention the polypeptides in which up to 5 amino acids can be modified, truncated at the N- or C-terminal end, or even deleted or added.

As is indicated, the modifications of the polypeptide will especially have as objective:

- to render it capable of modulating, of inhibiting or of inducing the expression of PWD circovirus gene and/or capable of modulating the replication cycle of PWD circovirus in the cell and/or the host organism,

- of allowing its incorporation into vaccine compositions,

5       - of modifying its bioavailability as a compound for therapeutic use.

The methods allowing said modulations on eukaryotic or prokaryotic cells to be demonstrated are well known to the person skilled in the art. It is likewise well understood that it will be possible to use the nucleotide sequences coding for said modified polypeptides for said modulations, for example through  
10       vectors according to the invention and described below, in order, for example, to prevent or to treat the pathologies linked to the infection.

The preceding modified polypeptides can be obtained by using combinatorial chemistry, in which it is possible to systematically vary parts of the polypeptide before testing them on models, cell cultures or microorganisms for example, to  
15       select the compounds which are most active or have the properties sought.

Chemical synthesis likewise has the advantage of being able to use:

- unnatural amino acids, or

- nonpeptide bonds.

Thus, in order to improve the duration of life of the polypeptides according  
20       to the invention, it may be of interest to use unnatural amino acids, for example in D form, or else amino acid analogs, especially sulfur-containing forms, for example.

Finally, it will be possible to integrate the structure of the polypeptides according to the invention, its specific or modified homologous forms, into chemical  
25       structures of polypeptide type or others. Thus, it may be of interest to provide at the N- and C-terminal ends compounds not recognized by the proteases.

The nucleotide sequences coding for a polypeptide according to the invention are likewise part of the invention.

The invention likewise relates to nucleotide sequences utilizable as a primer or probe, characterized in that said sequences are selected from the nucleotide sequences according to the invention.

5 Among the pairs of nucleotide sequences utilizable as a pair of primers according to the invention, the pairs of primers selected from the following pairs are preferred:

a) SEQ ID No. 46 5' GTG TGC TCG ACA TTG GTG TG 3', and

SEQ ID No. 47 5' TGG AAT GTT AAC GAG CTG AG 3';

10 ~~b) 5' GTG TGC TCG ACA TTG GTG TG 3', and~~

~~5' CTC GCA GCC ATC TTG GAA TG 3';~~

~~c) 5' CGC GCG TAA TAC GAC TCA CT 3', and~~

b) SEQ ID No. 46 5' GTG TGC TCG ACA TTG GTG TG 3',

and

15 ~~d) 5' CGC GCG TAA TAC GAC TCA CT 3', and~~

SEQ ID No. 48 5' CTC GCA GCC ATC TTG GAA TG 3';

and

c) SEQ ID No. 49 5' CGC GCG TAA TAC GAC TCA CT 3',

and

20 SEQ ID No. 46 5' GTG TGC TCG ACA TTG GTG TG 3';

d) SEQ ID No. 49 5' CGC GCG TAA TAC GAC TCA CT 3',

and

SEQ ID No. 48 5' CTC GCA GCC ATC TTG GAA TG 3';

and

25 e) SEQ ID No. 50 5' CCT GTC TAC TGC TGT GAG TAC CTT  
GT 3', and

SEQ ID No. 51 5' GCA GTA GAC AGG TCA CTC CGT  
TGT CC 3'.

30 The cloning and the sequencing of the PWD circovirus, type A and B, has allowed it to be identified, after comparative analysis with the nucleotide sequences of other porcine circoviruses, that, among the sequences of fragments of these nucleic acids, were those which are strictly specific to the PWD circovirus of type A, of type B or of type A and B, and those which correspond to a consensus



sequence of porcine circoviruses other than the PWD circoviruses of type A and/or B.

5 There is likewise a great need for nucleotide sequences utilizable as a primer or probe specific to the whole of the other known and nonpathogenic porcine circoviruses.

Said consensus nucleotide sequences specific to all circoviruses, other than PWD circovirus of type A and B, are easily identifiable from Figure 3 and the sequence SEQ ID No. ~~9~~715, and are part of the invention.

10 Among said consensus nucleotide sequences, that which is characterized in that it is part of the following pair of primers is preferred:

a) SEQ ID No. 46 5' GTG TGC TCG ACA TTG GTG TG 3', and

SEQ ID No. 52 5' TGG AAT GTT AAC TAC CTC AA 3' ~~+~~±

15 The invention likewise comprises a nucleotide sequence according to the invention, characterized in that said sequence is a specific consensus sequence of porcine circovirus other than PWD circovirus of type B and in that it is one of the primers of the following pairs of primers:

a) SEQ ID No. 53 5' GGC GGC GCC ATC TGT AAC GGT TT 3', and

20 SEQ ID No. 54 5' GAT GGC GCC GAA AGA CGG GTA TC 3' ~~+~~±

It is well understood that the present invention likewise relates to specific polypeptides of known porcine circoviruses other than PWD circovirus, encoded by said consensus nucleotide sequences, capable of being obtained by purification from natural polypeptides, by genetic recombination or by chemical  
25 synthesis by procedures well known to the person skilled in the art and such as described in particular below. In the same manner, the labeled or unlabeled mono- or polyclonal antibodies directed against said specific polypeptides encoded by said consensus nucleotide sequences are also part of the invention.

30 It will be possible to use said consensus nucleotide sequences, said corresponding polypeptides as well as said antibodies directed against said polypeptides in procedures or sets for detection and/or identification such as

described below, in place of or in addition to nucleotide sequences, polypeptides or antibodies according to the invention, specific to PWD circovirus type A and/or B.

5        These protocols have been improved for the differential detection of the circular monomeric forms of specific replicative forms of the virion or of the DNA in replication and the dimeric forms found in so-called in-tandem molecular constructs.

      The invention additionally relates to the use of a nucleotide sequence according to the invention as a primer or probe for the detection and/or the amplification of nucleic acid sequences.

10       The nucleotide sequences according to the invention can thus be used to amplify nucleotide sequences, especially by the PCR technique (polymerase chain reaction) (Erlich, 1989; Innis et al., 1990; Rolfs et al., 1991; and White et al., 1997).

15       These oligodeoxyribonucleotide or oligoribonucleotide primers advantageously have a length of at least 8 nucleotides, preferably of at least 12 nucleotides, and even more preferentially at least 20 nucleotides.

      Other amplification techniques of the target nucleic acid can be advantageously employed as alternatives to PCR.

20       The nucleotide sequences of the invention, in particular the primers according to the invention, can likewise be employed in other procedures of amplification of a target nucleic acid, such as:

- the TAS technique (Transcription-based Amplification System), described by Kwoh et al. in 1989;
- the 3SR technique (Self-Sustained Sequence Replication), described by  
25        Guatelli et al. in 1990;
- the NASBA technique (Nucleic Acid Sequence Based Amplification), described by Kievitis et al. in 1991;
- the SDA technique (Strand Displacement Amplification) (Walker et al., 1992);



- the TMA technique (Transcription Mediated Amplification).

The polynucleotides of the invention can also be employed in techniques of amplification or of modification of the nucleic acid serving as a probe, such as:

- 5       - the LCR technique (Ligase Chain Reaction), described by Landegren et al. in 1988 and improved by Barany et al. in 1991, which employs a thermostable ligase;
- the RCR technique (Repair Chain Reaction), described by Segev in 1992;
- the CPR technique (Cycling Probe Reaction), described by Duck et al. in  
10       1990;
- the amplification technique with Q-beta replicase, described by Miele et al. in 1983 and especially improved by Chu et al. in 1986, Lizardi et al. in 1988, then by Burg et al. as well as by Stone et al. in 1996.

15       In the case where the target polynucleotide to be detected is possibly an RNA, for example an mRNA, it will be possible to use, prior to the employment of an amplification reaction with the aid of at least one primer according to the invention or to the employment of a detection procedure with the aid of at least one probe of the invention, an enzyme of reverse transcriptase type in order to obtain a cDNA from the RNA contained in the biological sample. The cDNA obtained will  
20       thus serve as a target for the primer(s) or the probe(s) employed in the amplification or detection procedure according to the invention.

      The detection probe will be chosen in such a manner that it hybridizes with the target sequence or the amplicon generated from the target sequence. By way of sequence, such a probe will advantageously have a sequence of at least 12  
25       nucleotides, in particular of at least 20 nucleotides, and preferably of at least 100 nucleotides.

      The invention also comprises the nucleotide sequences utilizable as a probe or primer according to the invention, characterized in that they are labeled with a radioactive compound or with a nonradioactive compound.

The unlabeled nucleotide sequences can be used directly as probes or primers, although the sequences are generally labeled with a radioactive element ( $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^3\text{H}$ ,  $^{125}\text{I}$ ) or with a nonradioactive molecule (biotin, acetylaminofluorene, digoxigenin, 5-bromodeoxyuridine, fluorescein) to obtain probes which are  
5 utilizable for numerous applications.

Examples of nonradioactive labeling of nucleotide sequences are described, for example, in French Patent No. 78.10975 or by Urdea et al. or by Sanchez-Pescador et al. in 1988.

10 In the latter case, it will also be possible to use one of the labeling methods described in patents FR-2 422 956 and FR-2 518 755.

The hybridization technique can be carried out in various manners (Matthews et al., 1988). The most general method consists in immobilizing the nucleic acid extract of cells on a support (such as nitrocellulose, nylon, polystyrene) and in incubating, under well-defined conditions, the immobilized target nucleic acid with  
15 the probe. After hybridization, the excess of probe is eliminated and the hybrid molecules formed are detected by the appropriate method (measurement of the radioactivity, of the fluorescence or of the enzymatic activity linked to the probe).

The invention likewise comprises the nucleotide sequences according to the invention, characterized in that they are immobilized on a support, covalently or  
20 noncovalently.

According to another advantageous mode of employing nucleotide sequences according to the invention, the latter can be used immobilized on a support and can thus serve to capture, by specific hybridization, the target nucleic acid obtained from the biological sample to be tested. If necessary, the solid support is separated  
25 from the sample and the hybridization complex formed between said capture probe and the target nucleic acid is then detected with the aid of a second probe, a so-called detection probe, labeled with an easily detectable element.

Another subject of the present invention is a vector for the cloning and/or expression of a sequence, characterized in that it contains a nucleotide sequence according to the invention.

5 The vectors according to the invention, characterized in that they contain the elements allowing the expression and/or the secretion of said nucleotide sequences in a determined host cell, are likewise part of the invention.

10 The vector must then contain a promoter, signals of initiation and termination of translation, as well as appropriate regions of regulation of transcription. It must be able to be maintained stably in the host cell and can optionally have particular signals specifying the secretion of the translated protein. These different elements are chosen as a function of the host cell used. To this end, the nucleotide sequences according to the invention can be inserted into autonomous replication vectors within the chosen host, or integrated vectors of the chosen host.

15 Such vectors will be prepared according to the methods currently used by the person skilled in the art, and it will be possible to introduce the clones resulting therefrom into an appropriate host by standard methods, such as, for example, lipofection, electroporation and thermal shock.

The vectors according to the invention are, for example, vectors of plasmid or viral origin.

20 A preferred vector for the expression of polypeptides of the invention is baculovirus.

The vector pBS KS in which is inserted the in-tandem DNA sequence of the PWD circovirus type A (or DFP) as deposited at the CNCM on 3 July 1997, under the number I-1891, is likewise preferred.

25 These vectors are useful for transforming host cells in order to clone or to express the nucleotide sequences of the invention.

The invention likewise comprises the host cells transformed by a vector according to the invention.

These cells can be obtained by the introduction into host cells of a nucleotide sequence inserted into a vector such as defined above, then the culturing of said cells under conditions allowing the replication and/or expression of the transfected nucleotide sequence.

5           The host cell can be selected from prokaryotic or eukaryotic systems, such as, for example, bacterial cells (Olins and Lee, 1993), but likewise yeast cells (Buckholz, 1993), as well as animal cells, in particular the cultures of mammalian cells (Edwards and Aruffo, 1993), and especially Chinese hamster ovary (CHO) cells, but likewise the cells of insects in which it is possible to use procedures  
10       employing baculoviruses, for example (Luckow, 1993).

A preferred host cell for the expression of the proteins of the invention is constituted by sf9 insect cells.

A more preferred host cell according to the invention is E. coli, such as deposited at the CNCM on 3 July 1997, under the number I-1891.

15           The invention likewise relates to animals comprising one of said transformed cells according to the invention.

The obtainment of transgenic animals according to the invention overexpressing one or more of the genes of PWD circovirus or part of the genes will be preferably carried out in rats, mice or rabbits according to methods well  
20       known to the person skilled in the art, such as by viral or nonviral transfections. It will be possible to obtain the transgenic animals overexpressing one or more of said genes by transfection of multiple copies of said genes under the control of a strong promoter of ubiquitous nature, or selective for one type of tissue. It will likewise be possible to obtain the transgenic animals by homologous recombination in  
25       embryonic cell strains, transfer of these cell strains to embryos, selection of the affected chimeras at the level of the reproductive lines, and growth of said chimeras.

The transformed cells as well as the transgenic animals according to the invention are utilizable in procedures for preparation of recombinant polypeptides.

It is today possible to produce recombinant polypeptides in relatively large quantity by genetic engineering using the cells transformed by expression vectors according to the invention or using transgenic animals according to the invention.

5 The procedures for preparation of a polypeptide of the invention in recombinant form, characterized in that they employ a vector and/or a cell transformed by a vector according to the invention and/or a transgenic animal comprising one of said transformed cells according to the invention, are themselves comprised in the present invention.

10 Among said procedures for preparation of a polypeptide of the invention in recombinant form, the preparation procedures employing a vector, and/or a cell transformed by said vector and/or a transgenic animal comprising one of said transformed cells, containing a nucleotide sequence according to the invention coding for a polypeptide of PWD circovirus, are preferred.

15 The recombinant polypeptides obtained as indicated above can just as well be present in glycosylated form as in nonglycosylated form and can or cannot have the natural tertiary structure.

20 A preferred variant consists in producing a recombinant polypeptide ~~fused~~used to a "carrier" protein (chimeric protein). The advantage of this system is that it allows a stabilization of and a decrease in the proteolysis of the recombinant product, an increase in the solubility in the course of renaturation in vitro and/or a simplification of the purification when the fusion partner has an affinity for a specific ligand.

More particularly, the invention relates to a procedure for preparation of a polypeptide of the invention comprising the following steps:

- 25 a) culture of transformed cells under conditions allowing the expression of a recombinant polypeptide of nucleotide sequence according to the invention;  
b) if need be, recovery of said recombinant polypeptide.

When the procedure for preparation of a polypeptide of the invention employs a transgenic animal according to the invention, the recombinant polypeptide is then extracted from said animal.

5 The invention also relates to a polypeptide which is capable of being obtained by a procedure of the invention such as described previously.

The invention also comprises a procedure for preparation of a synthetic polypeptide, characterized in that it uses a sequence of amino acids of polypeptides according to the invention.

10 The invention likewise relates to a synthetic polypeptide obtained by a procedure according to the invention.

The polypeptides according to the invention can likewise be prepared by techniques which are conventional in the field of the synthesis of peptides. This synthesis can be carried out in homogeneous solution or in solid phase.

15 For example, recourse can be made to the technique of synthesis in homogeneous solution described by Houben-Weyl in 1974.

20 This method of synthesis consists in successively condensing, two by two, the successive amino acids in the order required, or in condensing amino acids and fragments formed previously and already containing several amino acids in the appropriate order, or alternatively several fragments previously prepared in this way, it being understood that it will be necessary to protect beforehand all the reactive functions carried by these amino acids or fragments, with the exception of amine functions of one and carboxyls of the other or vice-versa, which must normally be involved in the formation of peptide bonds, especially after activation of the carboxyl function, according to the methods well known in the synthesis of  
25 peptides.

According to another preferred technique of the invention, recourse will be made to the technique described by Merrifield.

To make a peptide chain according to the Merrifield procedure, recourse is made to a very porous polymeric resin, on which is immobilized the first C-terminal



amino acid of the chain. This amino acid is immobilized on a resin through its carboxyl group and its amine function is protected. The amino acids which are going to form the peptide chain are thus immobilized, one after the other, on the amino group, which is deprotected beforehand each time, of the portion of the peptide chain already formed, and which is attached to the resin. When the whole of the desired peptide chain has been formed, the protective groups of the different amino acids forming the peptide chain are eliminated and the peptide is detached from the resin with the aid of an acid.

The invention additionally relates to hybrid polypeptides having at least one polypeptide according to the invention, and a sequence of a polypeptide capable of inducing an immune response in man or animals.

Advantageously, the antigenic determinant is such that it is capable of inducing a humoral and/or cellular response.

It will be possible for such a determinant to comprise a polypeptide according to the invention in glycosylated form used with a view to obtaining immunogenic compositions capable of inducing the synthesis of antibodies directed against multiple epitopes. Said polypeptides or their glycosylated fragments are likewise part of the invention.

These hybrid molecules can be formed, in part, of a polypeptide carrier molecule or of fragments thereof according to the invention, associated with a possibly immunogenic part, in particular an epitope of the diphtheria toxin, the tetanus toxin, a surface antigen of the hepatitis B virus (patent FR 79 21811), the VP1 antigen of the poliomyelitis virus or any other viral or bacterial toxin or antigen.

The procedures for synthesis of hybrid molecules encompass the methods used in genetic engineering for constructing hybrid nucleotide sequences coding for the polypeptide sequences sought. It will be possible, for example, to refer advantageously to the technique for obtainment of genes coding for fusion proteins described by Minton in 1984.



Said hybrid nucleotide sequences coding for a hybrid polypeptide as well as the hybrid polypeptides according to the invention characterized in that they are recombinant polypeptides obtained by the expression of said hybrid nucleotide sequences are likewise part of the invention.

5           The invention likewise comprises the vectors characterized in that they contain one of said hybrid nucleotide sequences. The host cells transformed by said vectors, the transgenic animals comprising one of said transformed cells as well as the procedures for preparation of recombinant polypeptides using said vectors, said transformed cells and/or said transgenic animals are, of course, likewise part of the  
10           invention.

          The polypeptides according to the invention, the antibodies according to the invention described below and the nucleotide sequences according to the invention can advantageously be employed in procedures for the detection and/or identification of PWD circovirus, or of porcine circovirus other than a PWD  
15           circovirus, in a biological sample (biological tissue or fluid) capable of containing them. These procedures, according to the specificity of the polypeptides, the antibodies and the nucleotide sequences according to the invention which will be used, will in particular be able to detect and/or to identify a PWD circovirus or a porcine circovirus other than a PWD circovirus or other than the PWD circovirus of  
20           type B.

          The polypeptides according to the invention can advantageously be employed in a procedure for the detection and/or the identification of PWD circovirus of type A, of type B, of type A or B, or porcine circovirus other than the PWD circovirus of type B, or of porcine circovirus other than the PWD circovirus of type A or B, in  
25           a biological sample (biological tissue or fluid) capable of containing them, characterized in that it comprises the following steps:

- a) contacting of this biological sample with a polypeptide or one of its fragments according to the invention (under conditions allowing an

immunological reaction between said polypeptide and the antibodies possibly present in the biological sample);

b) demonstration of the antigen-antibody complexes possibly formed.

5 In the present description, PWD circovirus, except if a particular mention is indicated, will be understood as designating a PWD circovirus of type A or of type B, and porcine circovirus other than PWD, except if a particular mention is indicated, will be understood as designating a porcine circovirus other than a PWD circovirus of type A and B.

10 Preferably, the biological sample is formed by a fluid, for example a pig serum, whole blood or biopsies.

Any conventional procedure can be employed for carrying out such a detection of the antigen-antibody complexes possibly formed.

15 By way of example, a preferred method brings into play immunoenzymatic processes according to the ELISA technique, by immunofluorescence, or radioimmunological processes (RIA) or their equivalent.

Thus, the invention likewise relates to the polypeptides according to the invention, labeled with the aid of an adequate label such as of the enzymatic, fluorescent or radioactive type.

Such methods comprise, for example, the following steps:

- 20
- deposition of determined quantities of a polypeptide composition according to the invention in the wells of a microtiter plate,
  - introduction into said wells of increasing dilutions of serum, or of a biological sample other than that defined previously, having to be analyzed,
  - incubation of the microplate,
  - 25 - introduction into the wells of the microtiter plate of labeled antibodies directed against pig immunoglobulins, the labeling of these antibodies having been carried out with the aid of an enzyme selected from those which are capable of hydrolyzing a substrate by modifying the absorption of the

radiation of the latter, at least at a determined wavelength, for example at 550 nm,

- detection, by comparison with a control test, of the quantity of hydrolyzed substrate.

5

The invention likewise relates to a kit or set for the detection and/or identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:

10

- a polypeptide according to the invention,
- if need be, the reagents for the formation of the medium favorable to the immunological or specific reaction,
- if need be, the reagents allowing the detection of the antigen-antibody complexes produced by the immunological reaction between the polypeptide(s) of the invention and the antibodies possibly present in the biological sample, these reagents likewise being able to carry a label, or to be recognized in their turn by a labeled reagent, more particularly in the case where the polypeptide according to the invention is not labeled,
- if need be, a biological reference sample (negative control) devoid of antibodies recognized by a polypeptide according to the invention,
- if need be, a biological reference sample (positive control) containing a predetermined quantity of antibodies recognized by a polypeptide according to the invention.

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The polypeptides according to the invention allow monoclonal or polyclonal antibodies to be prepared which are characterized in that they specifically recognize the polypeptides according to the invention. It will advantageously be possible to prepare the monoclonal antibodies from hybridomas according to the technique described by Kohler and Milstein in 1975. It will be possible to prepare the polyclonal antibodies, for example, by immunization of an animal, in particular a mouse, with a polypeptide or a DNA, according to the invention, associated with an

adjuvant of the immune response, and then purification of the specific antibodies contained in the serum of the immunized animals on an affinity column on which the polypeptide which has served as an antigen has previously been immobilized. The polyclonal antibodies according to the invention can also be prepared by  
5 purification, on an affinity column on which a polypeptide according to the invention has previously been immobilized, of the antibodies contained in the serum of pigs infected by a PWD circovirus.

The invention likewise relates to mono- or polyclonal antibodies or their fragments, or chimeric antibodies, characterized in that they are capable of  
10 specifically recognizing a polypeptide according to the invention.

It will likewise be possible for the antibodies of the invention to be labeled in the same manner as described previously for the nucleic probes of the invention, such as a labeling of enzymatic, fluorescent or radioactive type.

The invention is additionally directed at a procedure for the detection and/or  
15 identification of PWD circovirus, of porcine circovirus other than a PWD circovirus, or other than the PWD circovirus of type B, in a biological sample, characterized in that it comprises the following steps:

a) contacting of the biological sample (biological tissue or fluid) with a mono- or polyclonal antibody according to the invention (under conditions allowing  
20 an immunological reaction between said antibodies and the polypeptides of PWD circovirus, of porcine circovirus other than a PWD circovirus, of porcine circovirus other than the PWD circovirus of type B, possibly present in the biological sample);

b) demonstration of the antigen-antibody complex possibly formed.

Likewise within the scope of the invention is a kit or set for the detection  
25 and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following components:

- a polyclonal or monoclonal antibody according to the invention, if need be labeled;

- if need be, a reagent for the formation of the medium favorable to the carrying out of the immunological reaction;
- if need be, a reagent allowing the detection of the antigen-antibody complexes produced by the immunological reaction, this reagent likewise being able to carry a label, or being capable of being recognized in its turn by a labeled reagent, more particularly in the case where said monoclonal or polyclonal antibody is not labeled;
- if need be, reagents for carrying out the lysis of cells of the sample tested.

10           The present invention likewise relates to a procedure for the detection and/or the identification of PWD, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, in a biological sample, characterized in that it employs a nucleotide sequence according to the invention.

15           More particularly, the invention relates to a procedure for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, in a biological sample, characterized in that it contains the following steps:

- a) if need be, isolation of the DNA from the biological sample to be analyzed;
- 20           b) specific amplification of the DNA of the sample with the aid of at least one primer, or a pair of primers, according to the invention;
- c) demonstration of the amplification products.

25           These can be detected, for example, by the technique of molecular hybridization utilizing a nucleic probe according to the invention. This probe will advantageously be labeled with a nonradioactive (cold probe) or radioactive element.

For the purposes of the present invention, "DNA of the biological sample" or "DNA contained in the biological sample" will be understood as meaning either the DNA present in the biological sample considered, or possibly the cDNA

obtained after the action of an enzyme of reverse transcriptase type on the RNA present in said biological sample.

Another aim of the present invention consists in a procedure according to the invention, characterized in that it comprises the following steps:

5           a)       contacting of a nucleotide probe according to the invention with a biological sample, the DNA contained in the biological sample having, if need be, previously been made accessible to hybridization under conditions allowing the hybridization of the probe with the DNA of the sample;

10           b)       demonstration of the hybrid formed between the nucleotide probe and the DNA of the biological sample.

The present invention also relates to a procedure according to the invention, characterized in that it comprises the following steps:

15           a)       contacting of a nucleotide probe immobilized on a support according to the invention with a biological sample, the DNA of the sample having, if need be, previously been made accessible to hybridization, under conditions allowing the hybridization of the probe with the DNA of the sample;

20           b)       contacting of the hybrid formed between the nucleotide probe immobilized on a support and the DNA contained in the biological sample, if need be after elimination of the DNA of the biological sample which has not hybridized with the probe, with a nucleotide probe labeled according to the invention;

          c)       demonstration of the novel hybrid formed in step b).

25           According to an advantageous embodiment of the procedure for detection and/or identification defined previously, this is characterized in that, prior to step a), the DNA of the biological sample is first amplified with the aid of at least one primer according to the invention.

The invention is additionally directed at a kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than the PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:



- a) a nucleotide probe according to the invention;
- b) if need be, the reagents necessary for the carrying out of a hybridization reaction;
- c) if need be, at least one primer according to the invention as well as the reagents necessary for an amplification reaction of the DNA.

5

The invention likewise relates to a kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following components:

- a) a nucleotide probe, called a capture probe, according to the invention;
- b) an oligonucleotide probe, called a revealing probe, according to the invention,
- c) if need be, at least one primer according to the invention, as well as the reagents necessary for an amplification reaction of the DNA.

10

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The invention also relates to a kit or set for the detection and/or identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:

- a) at least one primer according to the invention;
- b) if need be, the reagents necessary for carrying out a DNA amplification reaction;
- c) if need be, a component allowing the sequence of the amplified fragment to be verified, more particularly an oligonucleotide probe according to the invention.

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The invention additionally relates to the use of a nucleotide sequence according to the invention, of a polypeptide according to the invention, of an antibody according to the invention, of a cell according to the invention, and/or of an animal transformed according to the invention, for the selection of an organic or



inorganic compound capable of modulating, inducing or inhibiting the expression of genes, and/or of modifying the cellular replication of PWD circovirus or capable of inducing or of inhibiting the pathologies linked to an infection by a PWD circovirus.

5 The invention likewise comprises a method of selection of compounds capable of binding to a polypeptide or one of its fragments according to the invention, capable of binding to a nucleotide sequence according to the invention, or capable of recognizing an antibody according to the invention, and/or capable of modulating, inducing or inhibiting the expression of genes, and/or of modifying the cellular replication of PWD circovirus or capable of inducing or inhibiting the pathologies linked to an infection by a PWD circovirus, characterized in that it  
10 comprises the following steps:

a) contacting of said compound with said polypeptide, said nucleotide sequence, or with a cell transformed according to the invention and/or administration of said compound to an animal transformed according to the  
15 invention;

b) determination of the capacity of said compound to bind to said polypeptide or said nucleotide sequence, or to modulate, induce or inhibit the expression of genes, or to modulate the growth or the replication of PWD circovirus, or to induce or inhibit in said transformed animal the pathologies linked  
20 to an infection by PWD circovirus (designated activity of said compound).

The compounds capable of being selected can be organic compounds such as polypeptides or carbohydrates or any other organic or inorganic compounds already known, or novel organic compounds elaborated by molecular modelling techniques and obtained by chemical or biochemical synthesis, these techniques being known to  
25 the person skilled in the art.

It will be possible to use said selected compounds to modulate the cellular replication of PWD circovirus and thus to control infection by this virus, the methods allowing said modulations to be determined being well known to the person skilled in the art.

This modulation can be carried out, for example, by an agent capable of binding to a protein and thus of inhibiting or of potentiating its biological activity, or capable of binding to an envelope protein of the external surface of said virus and of blocking the penetration of said virus into the host cell or of favoring the action of the immune system of the infected organism directed against said virus. This modulation can likewise be carried out by an agent capable of binding to a nucleotide sequence of a DNA of said virus and of blocking, for example, the expression of a polypeptide whose biological or structural activity is necessary for the replication or for the proliferation of said virus host cells to host cells in the host animal.

The invention relates to the compounds capable of being selected by a selection method according to the invention.

The invention likewise relates to a pharmaceutical composition comprising a compound selected from the following compounds:

- a) a nucleotide sequence according to the invention;
  - b) a polypeptide according to the invention;
  - c) a vector, a viral particle or a cell transformed according to the invention;
  - d) an antibody according to the invention;
  - e) a compound capable of being selected by a selection method according to the invention;
- possibly in combination with a pharmaceutically acceptable vehicle and, if need be, with one or more adjuvants of the appropriate immunity.

The invention also relates to an immunogenic and/or vaccine composition, characterized in that it comprises a compound selected from the following compounds:

- a) a nucleotide sequence according to the invention;
- b) a polypeptide according to the invention;
- c) a vector or a viral particle according to the invention; and

d) a cell according to the invention.

~~The invention additionally relates to a~~ In one embodiment,  
the vaccine composition according to the invention<sub>7</sub> is characterized in that it  
comprises a mixture of at least two of said compounds a), b), c) and d) above and in  
5 that one of the two said compounds is related to the PWD circovirus of type A and  
the other is related to the PWD circovirus of type B.

In another embodiment of the invention, the vaccine composition is  
characterized in that it comprises at least one compound a), b), c), or d) above  
which is related to PWD circovirus of type B. In still another embodiment, the  
10 vaccine composition is characterized in that it comprises at least one compound a),  
b), c), or d) above which is related to PWD circovirus of type B ORF'2.

A compound related to the PWD circovirus of type A or of type B is  
understood here as respectively designating a compound obtained from the genomic  
sequence of the PWD circovirus of type A or of type B.

15 The invention is additionally aimed at an immunogenic and/or vaccine  
composition, characterized in that it comprises at least one of the following  
compounds:

- a nucleotide sequence SEQ ID No. 11<sub>7</sub>23, SEQ ID No. 12<sub>7</sub>25, or  
one of their fragments or homologues;
- 20 - a polypeptide of sequence SEQ ID No. 14<sub>7</sub>24, SEQ ID No. 15<sub>7</sub>26,  
or one of their fragments, or a modification thereof;
- a vector or a viral particle comprising a nucleotide sequence SEQ ID  
No. 11<sub>7</sub>23, SEQ ID No. 12<sub>7</sub>25, or one of their fragments or homologues;
- a transformed cell capable of expressing a polypeptide of sequence  
25 SEQ ID No. 14<sub>7</sub>24, SEQ ID No. 15<sub>7</sub>26, or one of their fragments, or a  
modification thereof; or
- a mixture of at least two of said compounds.

The invention also comprises an immunogenic and/or vaccine composition  
according to the invention, characterized in that it comprises said mixture of at least

two of said compounds as a combination product for simultaneous, separate or protracted use for the prevention or the treatment of infection by a PWD circovirus, especially of type B.

In a preferred embodiment, the vaccine composition according to the invention comprises the mixture of the following compounds:

- a pcDNA3 plasmid containing a nucleic acid of sequence SEQ ID No. 1123;
- a pcDNA3 plasmid containing a nucleic acid of sequence SEQ ID No. 1225;
- a pcDNA3 plasmid containing a nucleic acid coding for the GM-CSF protein;
- a recombinant baculovirus containing a nucleic acid of sequence SEQ ID No. 1123;
- a recombinant baculovirus containing a nucleic acid of sequence SEQ ID No. 1225; and
- if need be, an adjuvant of the appropriate immunity, especially the adjuvant AIF<sup>TM</sup>.

The invention is likewise directed at a pharmaceutical composition according to the invention, for the prevention or the treatment of an infection by a PWD circovirus.

The invention is also directed at a pharmaceutical composition according to the invention for the prevention or the treatment of an infection by the PWD circovirus of type B.

The invention likewise concerns the use of a composition according to the invention, for the preparation of a medicament intended for the prevention or the treatment of infection by a PWD circovirus, preferably by the PWD circovirus of type B.

Under another aspect, the invention relates to a vector, a viral particle or a cell according to the invention, for the treatment and/or the prevention of a disease by gene therapy.

Finally, the invention comprises the use of a vector, of a viral particle or of a cell according to the invention for the preparation of a medicament intended for the treatment and/or the prevention of a disease by gene therapy.

The polypeptides of the invention entering into the immunogenic or vaccine compositions according to the invention can be selected by techniques known to the person skilled in the art such as, for example, depending on the capacity of said polypeptides to stimulate the T cells, which is translated, for example, by their proliferation or the secretion of interleukins, and which leads to the production of antibodies directed against said polypeptides.

In pigs, as in mice, in which a weight dose of the vaccine composition comparable to the dose used in man is administered, the antibody reaction is tested by taking of the serum followed by a study of the formation of a complex between the antibodies present in the serum and the antigen of the vaccine composition, according to the usual techniques.

The pharmaceutical compositions according to the invention will contain an effective quantity of the compounds of the invention, that is to say in sufficient quantity of said compound(s) allowing the desired effect to be obtained, such as, for example, the modulation of the cellular replication of PWD circovirus. The person skilled in the art will know how to determine this quantity, as a function, for example, of the age and of the weight of the individual to be treated, of the state of advancement of the pathology, of the possible secondary effects and by means of a test of evaluation of the effects obtained on a population range, these tests being known in these fields of application.

According to the invention, said vaccine combinations will preferably be combined with a pharmaceutically acceptable vehicle and, if need be, with one or more adjuvants of the appropriate immunity.

Today, various types of vaccines are available for protecting animals or man against infectious diseases: attenuated living microorganisms (*M. bovis* - BCG for tuberculosis), inactivated microorganisms (influenza virus), acellular extracts (*Bordetella pertussis* for whooping cough), recombined proteins (surface antigen of the hepatitis B virus), polysaccharides (pneumococcal). Vaccines prepared from synthetic peptides or genetically modified microorganisms expressing heterologous antigens are in the course of experimentation. More recently still, recombined plasmid DNAs carrying genes coding for protective antigens have been proposed as an alternative vaccine strategy. This type of vaccination is carried out with a particular plasmid originating from a plasmid of *E. coli* which does not replicate *in vivo* and which codes uniquely for the vaccinating protein. Animals have been immunized by simply injecting the naked plasmid DNA into the muscle. This technique leads to the expression of the vaccine protein *in situ* and to an immune response of cellular type (CTL) and of humoral type (antibody). This double induction of the immune response is one of the principal advantages of the vaccination technique with naked DNA.

The vaccine compositions comprising nucleotide sequences or vectors into which are inserted said sequences are especially described in the international application No. WO 90/11092 and likewise in the international application No. WO 95/11307.

The constitutive nucleotide sequence of the vaccine composition according to the invention can be injected into the host after having been coupled to compounds which favor the penetration of this polynucleotide into the interior of the cell or its transport to the cell nucleus. The resultant conjugates can be encapsulated in polymeric microparticles, as described in the international application No. WO 94/27238 (Medisorb Technologies International).

According to another embodiment of the vaccine composition according to the invention, the nucleotide sequence, preferably a DNA, is complexed with DEAE-dextran (Pagano et al., 1967) or with nuclear proteins



(Kaneda et al., 1989), with lipids (Felgner et al., 1987) or encapsulated in liposomes (Fraley et al., 1980) or else introduced in the form of a gel facilitating its transfection into the cells (Midoux et al., 1993, Pastore et al., 1994). The polynucleotide or the vector according to the invention can also be in suspension in  
5 a buffer solution or be combined with liposomes.

Advantageously, such a vaccine will be prepared according to the technique described by Tacson et al. or Huygen et al. in 1996 or alternatively according to the technique described by Davis et al. in the international application No. WO 95/11307.

10 Such a vaccine can likewise be prepared in the form of a composition containing a vector according to the invention, placed under the control of regulation elements allowing its expression in man or animal. It will be possible, for example, to use, by way of *in vivo* expression vector of the polypeptide antigen of interest, the plasmid pcDNA3 or the plasmid pcDNA1/neo, both marketed by  
15 Invitrogen (R&D Systems, Abingdon, United Kingdom). It is also possible to use the plasmid V1Jns.tPA, described by Shiver et al. in 1995. Such a vaccine will advantageously comprise, apart from the recombinant vector, a saline solution, for example a sodium chloride solution.

Pharmaceutically acceptable vehicle is understood as designating a compound  
20 or a combination of compounds entering into a pharmaceutical composition or vaccine which does not provoke secondary reactions and which allows, for example, the facilitation of the administration of the active compound, an increase in its duration of life and/or its efficacy in the body, an increase in its solubility in solution or alternatively an improvement in its conservation. These pharmaceutically  
25 acceptable vehicles are well known and will be adapted by the person skilled in the art as a function of the nature and of the mode of administration of the chosen active compound.

As far as the vaccine formulations are concerned, these can comprise adjuvants of the appropriate immunity which are known to the person skilled in the



art, such as, for example, aluminum hydroxide, a representative of the family of muramyl peptides such as one of the peptide derivatives of N-acetyl muramyl, a bacterial lysate, or alternatively Freund's incomplete adjuvant.

5 These compounds can be administered by the systemic route, in particular by the intravenous route, by the intramuscular, intradermal or subcutaneous route, or by the oral route. In a more preferred manner, the vaccine composition comprising polypeptides according to the invention will be administered by the intramuscular route, through the food or by nebulization several times, staggered over time.

10 Their administration modes, dosages and optimum pharmaceutical forms can be determined according to the criteria generally taken into account in the establishment of a treatment adapted to an animal such as, for example, the age or the weight, the seriousness of its general condition, the tolerance to the treatment and the secondary effects noted. Preferably, the vaccine of the present invention is administered in an amount that is protective against piglet weight loss disease.

15 For example, in the case of a vaccine according to the present invention comprising a polypeptide encoded by a nucleotide sequence of the genome of PCV, or a homologue or fragment thereof, the polypeptide will be administered one time or several times, spread out over time, directly or by means of a transformed cell capable of expressing the polypeptide, in an amount of about 0.1 to 10 µg per  
20 kilogram weight of the animal, preferably about 0.2 to about 5 µg/kg, more preferably about 0.5 to about 2 µg/kg for a dose.

25 The present invention likewise relates to the use of nucleotide sequences of PWD circovirus according to the invention for the construction of autoreplicative retroviral vectors and the therapeutic applications of these, especially in the field of human gene therapy in vivo.

The feasibility of gene therapy applied to man no longer needs to be demonstrated and this relates to numerous therapeutic applications like genetic diseases, infectious diseases and cancers. Numerous documents of the prior art describe the means of employing gene therapy, especially through viral vectors.

Generally speaking, the vectors are obtained by deletion of at least some of the viral genes which are replaced by the genes of therapeutic interest. Such vectors can be propagated in a complementation line which supplies in trans the deleted viral functions in order to generate a defective viral vector particle for replication but  
5 capable of infecting a host cell. To date, the retroviral vectors are amongst the most widely used and their mode of infection is widely described in the literature accessible to the person skilled in the art.

The principle of gene therapy is to deliver a functional gene, called a gene of interest, of which the RNA or the corresponding protein will produce the desired  
10 biochemical effect in the targeted cells or tissues. On the one hand, the insertion of genes allows the prolonged expression of complex and unstable molecules such as RNAs or proteins which can be extremely difficult or even impossible to obtain or to administer directly. On the other hand, the controlled insertion of the desired gene into the interior of targeted specific cells allows the expression product to be  
15 regulated in defined tissues. For this, it is necessary to be able to insert the desired therapeutic gene into the interior of chosen cells and thus to have available a method of insertion capable of specifically targeting the cells or the tissues chosen.

Among the methods of insertion of genes, such as, for example, microinjection, especially the injection of naked plasmid DNA (Derse, D. et al.,  
20 1995, and Zhao, T.M. et al., 1996), electroporation, homologous recombination, the use of viral particles, such as retroviruses, is widespread. However, applied in vivo, the gene transfer systems of recombinant retroviral type at the same time have a weak infectious power (insufficient concentration of viral particles) and a lack of specificity with regard to chosen target cells.

25 The production of cell-specific viral vectors, having a tissue-specific tropism, and whose gene of interest can be translated adequately by the target cells, is realizable, for example, by fusing a specific ligand of the target host cells to the N-terminal part of a surface protein of the envelope of PWD circovirus. It is possible to mention, for example, the construction of retroviral particles having the

CD4 molecule on the surface of the envelope so as to target the human cells infected by the HIV virus (YOUNG, J.A.T. et al., Sciences 1990, 250, 1421-1423), viral particles having a peptide hormone fused to an envelope protein to specifically infect the cells expressing the corresponding receptor (KASAHARA, N. et al., Sciences 5 1994, 266, 1373-1376) or else alternatively viral particles having a fused polypeptide capable of immobilizing on the receptor of the epidermal growth factor (EGF) (COSSET, F.L. et al., J. of Virology 1995, 69, 10, 6314-6322). In another approach, single-chain fragments of antibodies directed against surface antigens of the target cells are inserted by fusion with the N-terminal part of the envelope protein (VALSESIA-WITTMAN, S. et al., J. of Virology 1996, 70, 3, 2059-2064; 10 TEARINA CHU, T.H. et al., J. of Virology 1997, 71, 1, 720-725).

For the purposes of the present invention, a gene of interest in use in the invention can be obtained from a eukaryotic or prokaryotic organism or from a virus by any conventional technique. It is, preferably, capable of producing an expression 15 product having a therapeutic effect and it can be a product homologous to the cell host or, alternatively, heterologous. In the scope of the present invention, a gene of interest can code for an (i) intracellular or (ii) membrane product present on the surface of the host cell or (iii) secreted outside the host cell. It can therefore comprise appropriate additional elements such as, for example, a sequence coding 20 for a secretion signal. These signals are known to the person skilled in the art.

In accordance with the aims pursued by the present invention, a gene of interest can code for a protein corresponding to all or part of a native protein as found in nature. It can likewise be a chimeric protein, for example arising from the fusion of polypeptides of various origins or from a mutant having improved and/or 25 modified biological properties. Such a mutant can be obtained, by conventional biological techniques, by substitution, deletion and/or addition of one or more amino acid residues.

It is very particularly preferred to employ a gene of therapeutic interest coding for an expression product capable of inhibiting or retarding the establishment

and/or the development of a genetic or acquired disease. A vector according to the invention is in particular intended for the prevention or for the treatment of cystic fibrosis, of hemophilia A or B, of Duchenne's or Becker's myopathy, of cancer, of AIDS and of other bacteria or infectious diseases due to a pathogenic organism: virus, bacteria, parasite or prion. The genes of interest utilizable in the present invention are those which code, for example, for the following proteins:

- a cytokine and especially an interleukin, an interferon, a tissue necrosis factor and a growth factor and especially a hematopoietic growth factor (G-CSF, GM-CSF),
- 10 - a factor or cofactor involved in clotting and especially factor VIII, von Willebrand's factor, antithrombin III, protein C, thrombin and hirudin,
- an enzyme or an enzyme inhibitor such as the inhibitors of viral proteases,
- an expression product of a suicide gene such as thymidine kinase of the HSV virus (herpesvirus) of type 1,
- 15 - an activator or an inhibitor of ion channels,
- a protein of which the absence, the modification or the deregulation of expression is responsible for a genetic disease, such as the CFTR protein, dystrophin or minidystrophin, insulin, ADA (adenosine diaminase), glucocerebrosidase and phenylhydroxylase,
- 20 - a protein capable of inhibiting the initiation or the progression of cancers, such as the expression products of tumor suppressor genes, for example the P53 and Rb genes,
- a protein capable of stimulating an immune or an antibody response, and
- a protein capable of inhibiting a viral infection or its development, for example the antigenic epitopes of the virus in question or altered variants of
- 25 viral proteins capable of entering into competition with the native viral proteins.

The invention thus relates to the vectors characterized in that they comprise a nucleotide sequence of PWD circovirus according to the invention, and in that they additionally comprise a gene of interest.

5 The present invention likewise relates to viral particles generated from said vector according to the invention. It additionally relates to methods for the preparation of viral particles according to the invention, characterized in that they employ a vector according to the invention, including viral pseudoparticles (VLP, virus-like particles).

10 The invention likewise relates to animal cells transfected by a vector according to the invention.

Likewise comprised in the invention are animal cells, especially mammalian, infected by a viral particle according to the invention.

15 The present invention likewise relates to a vector, a viral particle or a cell according to the invention, for the treatment and/or the prevention of a genetic disease or of an acquired disease such as cancer or an infectious disease. The invention is likewise directed at a pharmaceutical composition comprising, by way of therapeutic or prophylactic agent, a vector or a cell according to the invention, in combination with a vehicle acceptable from a pharmaceutical point of view.

20 Other characteristics and advantages of the invention appear in the examples and the following figures:.

Legends to the figures:

Figure 1: ~~Experimental scheme which has made it possible to bring about the isolation and the identification of the circovirus associated with PWD of type A and B.~~

25 ~~Test 1: experimental reproduction of the PWD by inoculation of pig organ homogenates from farms affected by PWD.~~

~~Test 2: experimental reproduction of PWD.~~

~~Test 3: experimental reproduction of PWD.~~

30 ~~Test 4: no experimental reproduction of PWD.~~

~~Figure 2: Organization of the genome of the circovirus associated with PWD of type A (PCVA)~~

~~— strand of (+) polarity (SEQ ID No. 1);~~

~~— strand of (-) polarity (SEQ ID No. 2, represented according to the orientation 3' → 5');~~

~~— sequences~~

The invention is described in more detail in the following illustrative examples. Although the examples may represent only selected embodiments of  
~~amino acids of proteins encoded by the two DNA strands in the three possible reading frames.~~

~~Figure 3: Alignment of the nucleotide sequence SEQ ID No. 1 of the PWD circovirus of type A (PCVA) and of the MEEHAN strain and MANKERTZ strain circoviruses of the porcine cell lines~~  
invention, it should be understood that the following examples are illustrative and not limiting.

~~Figure 4: Alignment of the sequence of amino acids SEQ ID No. 6 of a polypeptide encoded by the nucleotide sequence SEQ ID No. 3 (ORF1) of the PWD circovirus of type A (PCVA) and of corresponding nucleotide sequences of the MEEHAN strain and MANKERTZ strain circoviruses of the porcine cell lines.~~

~~Figure 5: Alignment of the sequence of amino acids SEQ ID No. 7 of a polypeptide encoded by the nucleotide sequence SEQ ID No. 4 (ORF2) of the PWD circovirus of type A (PCVA) and of corresponding nucleotide sequences of the MEEHAN strain and MANKERTZ strain circoviruses of the porcine cell lines.~~

~~Figure 6: Alignment of the sequence of amino acids SEQ ID No. 8 of a polypeptide encoded by the nucleotide sequence SEQ ID No. 5 (ORF3) of the PWD circovirus of type A (PCVA) and of corresponding nucleotide sequences of the MEEHAN strain and MANKERTZ strain circoviruses of the porcine cell lines.~~



~~Figure 7: Western blot analysis of recombinant proteins of the PWD circovirus of type A (PCVA).~~

~~The analyses were carried out on cell extracts of Sf9 cells obtained after infection with recombinant baculovirus PCF ORF 1.~~

~~Figure 8: Organization of the genome of the circovirus associated with the PWD of type B (PCVB)~~

~~— strand of (+) polarity (SEQ ID No. 9);~~

~~— strand of (-) polarity (SEQ ID No. 10, represented according to the orientation 3' → 5');~~

~~— sequence of amino acids of proteins encoded by the two DNA strands in the three possible reading frames.~~

~~Figure 9: Evolution of the daily mean gain (DMG) of pig farms affected by piglet weight loss disease (PWD), placed under experimental conditions.~~

~~Figure 10: DMG compared for the 3 batches of pigs (F1, F3 and F4) calculated over a period of 28 days, after vaccination test.~~

~~Figure 11: Hyperthermia greater than 41°C, expressed as a percentage compared for the 3 batches of pigs (F1, F3 and F4) calculated per week over a period of 28 days, after vaccination test.~~

~~Figure 12: Membranes of peptide spots corresponding to the ORF2s revealed with the aid of an infected pig serum, originating from a conventional farm.~~

~~The numbers of specific peptides of the circovirus of type B as well as their nonreactive homologs (type A) are indicated in bold.~~

~~The nonspecific immunogenic peptides are indicated in italics.~~

~~Figure 13: Alignment of amino acid sequences of proteins encoded by the ORF2 of the PWD circovirus of type A and by the ORF'2 of the PWD circovirus of type B. The~~



position of 4 peptides corresponding to specific epitopes  
of the PWD circovirus of type B is indicated on the  
corresponding sequence by a bold line, their homolog on  
the sequence of the PWD circovirus of type A is likewise  
indicated by an ordinary line.

### Examples

#### EXAMPLES

10 EXAMPLE 1: Cloning, sequencing and characterization of the PWD circovirus of  
type A (PCVA)

1 1. Experimental procedures

Experimental reproduction of the infection and its syndrome (cf. Figure 1).

15 A first test was carried out with pigs from a very well-kept farm, but  
affected by piglet weight loss disease (PWD), likewise called fatal piglet wasting  
(FPW). Tests carried out with SPF (specific pathogen-free) pigs showed a transfer  
of contaminant(s) finding expression in a complex pathology combining  
hyperthermia, retardation of growth, diarrhea and conjunctivitis. The PDRS  
(porcine dysgenic and respiratory syndrome) virus, an infectious disease due to an  
20 arteriovirus) was rapidly isolated from breeding pigs and contact pigs. It should  
have been possible to attribute all the clinical signs to the presence of the PDRS  
virus. However, two farm pigs presented signs of FPW without the PDRS virus  
being isolated. The histological analyses and blood formulas, however, showed that  
these pigs were suffering from an infectious process of viral origin.

25 In a second test, 8-week SPF pigs were inoculated by the intratracheal route  
with organ homogenates of two farm pigs suffering from FPW. The inoculated pigs  
exhibited hyperthermia 8 to 9 days post-infection, then their growth was retarded.  
Other SPF pigs, placed in contact, had similar, attenuated signs 30 days after the  
initial experiment. No seroconversion with respect to a European or Canadian strain  
30 of PDRS virus was recorded in these animals.

A third test allowed the syndrome to be reproduced from samples taken from  
the pigs of the second test.

#### Conclusion

The syndrome is reproduced under the experimental conditions. It is determined by at least one infectious agent, which is transmittable by direct contact. The clinical constants are a sometimes high hyperthermia (greater than or equal to 41.5°C) which develops 8 to 10 days after infection. Retardation of the growth can be observed. The other signs are a reversal of the blood formula (reversal of the lymphocyte/polynuclear ratio from 70/30 to 30/70) and frequent lesions on the ganglia, especially those draining the respiratory apparatus (ganglionic hypertrophy, loss of structure with necrosis and infiltration by mononucleated or plurinucleated giant cells).

#### 2. Laboratory studies

Various cell supports including primary pig kidney cells or cell lines, pig testicle cells, monkey kidney cells, pig lymphocytes, pig alveolar macrophages and circulating blood monocytes were used to demonstrate the possible presence of a virus. No cytopathic effect was demonstrated in these cells. On the other hand, the use of a serum of a pig sick after experimental infection allowed an intracellular antigen to be revealed in the monocytes, the macrophages and approximately 10% of pig kidney (PK) cells infected with organ homogenates. This indirect revealing was carried out kinetically at different culture times. It is evident from this that the antigen initially appears in the nucleus of the infected cells before spreading into the cytoplasm. The successive passages in cell culture did not allow the signal to be amplified.

Under electron microscopy on organ homogenates, spherical particles labeled specifically by the serum of sick pigs, infected under the experimental conditions, were visualized. The size of these particles is estimated at 20 nm.

After two passages of these organ homogenates over pig lymphocytes and then three passages over pig kidney or testicle cells, a cytopathic effect developed and was amplified. An adenovirus was visualized in the electron microscope, which, under the experimental conditions, did not reproduce FPW (only a hyperthermia peak was noted 24 to 48 hours after infection, and then nothing more).

It has been possible to demonstrate DNA bands in certain samples of pigs infected under the experimental conditions and having exhibited signs of the disease (results not shown). A certain connection exists between the samples giving a positive result in cell culture and those having a DNA band.

#### Conclusion

At least two types of virus were demonstrated in the organ homogenates from pigs suffering from FPW. One is an adenovirus, but by itself alone it does not reproduce the disease. The other type of virus is a circovirus and is associated with FPW. This circovirus, of which two types have been isolated and sequenced, designated below PWD circovirus type A (or PCVA) and PWD circovirus of type B (or PCVB) have mutations with respect to the known sequences of circovirus which are nonpathogenic for the pig.

~~3~~ — 3. Cloning and sequencing of the DNA of the PWD circovirus of type A

Extraction of the replicative form (RF) DNA, cleavage by the Kpn I enzyme and amplification by a pair of primers flanking the Kpn I restriction site. Sequencing of the two strands at least twice by the Sanger method.

The nucleic sequence of the strand of (+) polarity of the genome of the PWD circovirus of type A (or PCVA), strain FPW, is represented by the sequence SEQ ID No. 1 in the list of sequences, the nucleic sequence of the strand of (-) polarity of the genome of the PWD circovirus of type A (or PCVA) being represented by the nucleic sequence 3' → 5' of Figure 3 or by the sequence SEQ ID No. ~~2~~5 (represented according to the orientation 5' → 3') in the list of sequences.

The amino acid sequences SEQ ID No. ~~6~~7, SEQ ID No. ~~7~~12 and SEQ ID No. ~~8~~14 of the list of sequences respectively represent the sequences of proteins encoded by the nucleic sequences of the 3 open reading frames SEQ ID No. ~~3~~9 (ORF1), corresponding to the REP protein, SEQ ID No. ~~4~~11 (ORF2) and SEQ ID No. ~~5~~13 (ORF3), determined from the sequence SEQ ID No. 1 of the strand of (+) polarity or of the nucleic sequence SEQ ID No. ~~2~~5 of the strand of (-) polarity of the genome of the PWD circovirus of type A.

~~4~~ — 4. Comparison of the nucleotide sequences and amino acids of the PWD circovirus of type A (or associated with PWD) which are obtained with the corresponding sequences of MEEHAN and MANKERTZ circoviruses of porcine cell lines

Use of the DNA sequence analysis software, DNASIS.

Sequences of oligonucleotides used as primers or probes in the detection and/or identification procedures

~~1. — specific~~ Specific detection of the PWD circovirus of type A:

SEQ ID No. 46 primer PCV 5: 5' GTG TGC TCG ACA TTG GTG TG 3';

SEQ ID No. 47 primer PCV 10: 5' TGG AAT GTT AAC GAG CTG AG 3';

2.—~~specific~~ Specific detection of the circovirus of the cell lines:

SEQ ID No. 46 primer PCF 5: 5' GTG TGC TCG ACA TTG GTG TG 3';

SEQ ID No. 52 primer MEE 1: 5' TGG AAT GTT AAC TAC CTC AA 3';

3.—~~differential~~ Differential detection:

5 the pairs of primers used are those described, for example, in the paragraphs 1 and 2 above;

4.—~~detection~~ Detection of the monomeric circular replicative forms RF:

SEQ ID No. 46 primer PCV 5: 5' GTG TGC TCG ACA TTG GTG TG 3';

SEQ ID No. 48 primer PCV 6: 5' CTC GCA GCC ATC TTG GAA TG 3';

10 5.—~~detection~~ Detection of the vectors carrying the dimers in tandem:

Nar dimer:

SEQ ID No. 49 primer KS 620: 5' CGC GCG TAA TAC GAC TCA CT 3';

SEQ ID No. 46 primer PCV 5: 5' GTG TGC TCG ACA TTG GTG TG 3';

Kpn dimer:

15 SEQ ID No. 49 primer KS 620: 5' CGC GCG TAA TAC GAC TCA CT 3';

SEQ ID No. 48 primer PCV 6: 5' CTC GCA GCC ATC TTG GAA TG 3';

6.—~~differential~~ Differential detection:

~~the~~The pairs of primers used are those described, for example, in paragraphs 4 and 5 above.

20 The procedures using the pairs or primers described in paragraphs 4 and 5 are of particular interest for differentially detecting the circular monomeric forms of specific replicative forms of the virion or of the DNA in replication and the dimeric forms found in the so-called in-tandem molecular constructs.

25 The in-tandem constructs of the viral genome (dimers) such as the constructs used for the preparation of the pBS KS + tandem PCV Kpn I vector, deposited at the CNCM under the number I-1891, 3 July 1997 (E. coli transformed by said vector) are very interesting for their use in methods of production in sufficient quantity of an inoculum formed of DNA, intended for the virus production, this in the absence of a satisfactory virus production protocol in a cell system. These said

methods of production using these in-tandem constructs of the viral genome will allow the virulence factors to be studied by mutation and by way of consequence will be able to be used for the production of a collection of viruses carrying the mutations indicated in the construction of vectors which will have the appropriate tropism and virulence. These vectors with autoreplicative structure have the sought gene transfer properties, especially for their applications in gene therapy, and in vaccinology.

Western-blot analysis of recombinant proteins of the PWD circovirus of type A

The results were obtained using a specific antiserum of the PWD circovirus produced during test 1 (cf. Figure 1).

Type of products analyzed.

The analyses were carried out on cell extracts of Sf9 cells obtained after infection by the recombinant baculovirus PCV ORF 1.

The culture of Sf9 cells was carried out in a 25 cm<sup>2</sup> Petri dish according to the standard culture methods for these cells. After centrifugation, the cell pellets are taken up with 300 µl of PBS buffer (phosphate saline buffer).

Electrophoresis (PAGE-SDS)

The electrophoresis is carried out on the cell extracts of Sf9 cells obtained previously on 5 samples (cf. Table 1 below) under the following conditions:

% polyacrylamide gel: 8%; conditions: denaturing

Voltage: 80 V; duration: 135 mn.

Table 1: Nature of the samples subjected to electrophoresis

Well No.	1	2	3	4	5
Sample applied	PM Rainbow	Raoul 24 h	Raoul 48 h	Raoul 72 h	Raoul 96 h
µl of sample	10	15	15	15	15
µl of Laemmli 4X	0	5	5	5	5

Legends to Table 1:

Laemmli 4X: loading buffer

PM Rainbow: molecular-weight markers (35, 52, 77, 107, 160 and 250 kD)

Raoul 24 h, 48 h, 72 h and 96 h: expression products of the ORF1 of the PWD circovirus of type A.

5

#### Western blot

After electrophoresis, the bands obtained in the different wells are transferred to nitrocellulose membrane for 1 h at 100 v in a TGM buffer (tris-glycine-methanol).

10

The Western blot is carried out under the following conditions:

1) Saturation with a solution containing 5% of skimmed milk; 0.05% of Tween 20 in a TBS 1X buffer (tris buffer saline) for 30 min.

2) 1st antibody:

15

10 ml of PWD anticircovirus antibody of type A are added diluted to 1/100, then the reaction mixture is incubated for one night at 4°C. Three washes of 10 min in TBS 1X are carried out.

3) 2nd antibody:

20

10 ml of pig rabbit P164 antibody anti-immunoglobulins, coupled to peroxidase (Dakopath) are added diluted to 1/100, then the reaction medium is incubated for 3 hours at 37°C. Three washes of 10 min in TBS 1X are carried out.

4) Visualization

The substrate 4-chloro-1-naphthol in the presence of oxygenated water is used for visualization.

25

#### Results

The results are shown in Figure 7.

30

Kinetics of appearance of antibodies specific for the REP recombinant protein of the PWD circovirus of type A expressed in baculovirus after infection of pigs by the PWD circovirus of type A (test 4, cf. Figure 1)



After infection of the pigs, a sample of serum of each of the infected pigs is taken at different periods expressed in the table by the date of taking (carried out here in the same year) and is then analyzed by Western blot.

5 The visualization of the specific antibodies is carried out in the manner described previously.

The results obtained are shown by Table 2 below.

Table 2: Kinetics of appearance of specific antibodies

Sample	Pigs	10/6	16/06	23/06	01/07	08/07	15/07	21/07
A3	1						Neg.	
Control	2						Neg.	
B2 Infec.	1	Neg.	Neg.	Neg.	+	+	++	+++
RP+	2	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
	3	Neg.	Neg.	Neg.	Neg.	+	+	+
	4	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	++

Legends to Table 2:

- 10 A3 control: uninfected control animals;  
 B2 Infec. RP+: animals infected with pig kidney (PK) cells containing the circovirus;  
 Neg.: negative;  
 +, ++, +++: intensity scale of the positive reaction;  
 15 10/06, 16/06, 23/06, 01/07, 08/07, 15/07, 21/07: dates expressed in day/month on which the different withdrawals of serum were carried out.

EXAMPLE 2: Cloning, sequencing and characterization of the type B PWD circovirus (PCVB)

- 20 The techniques used for cloning, sequencing and characterization of the type B PWD circovirus (PCVB) are those used in Example 1 above for the type A PWD circovirus (PCVA).

- The nucleic sequence of the strand of (+) polarity of the genome of the PWD circovirus of type B (or PCVB) is represented by the sequence SEQ ID No. 915 in the sequence listing, the nucleic sequence of the strand of (-) polarity of the genome of the PWD circovirus of type B (or PCVB) being represented by the



nucleic sequence 3' → 5' of Figure 8 or by the sequence SEQ ID No. ~~10~~19 (represented according to the orientation 5' → 3') in the sequence listing.

The amino acid sequences SEQ ID No. ~~14~~24, SEQ ID No. ~~15~~26 and SEQ ID No. ~~16~~28 of the sequence listing respectively represent the sequences of the proteins encoded by the nucleic sequences of the 3 open reading frames SEQ ID No. ~~11~~23 (ORF'1), corresponding to the REP protein, SEQ ID No. ~~12~~25 (ORF'2) and SEQ ID No. ~~13~~27 (ORF'3), determined from the sequence SEQ ID No. ~~9~~15 of the strand of (+) polarity or from the nucleic sequence SEQ ID No. ~~10~~19 of the strand of (-) polarity of the genome of the PWD circovirus of type B.

EXAMPLE 3: Comparative analysis of nucleotide sequences (ORF1, ORF2 and genomic) and amino acid sequences encoded by the ORF1 and the ORF2 of the PWD circoviruses of type A (PCVA) and of type B (PCVB)

The results expressed in % of homology are shown in Tables 3 and 4 below.

Table 3: Compared analysis of the amino acid sequences

% homology	ORF1	ORF2
PCVA/PCVB	80.4	56.2

Table 4: Compared analysis of the nucleotide sequences

% homology	Genomic	ORF1	ORF2	The remainder
PCVA/PCVB	70.4	80.4	60.1	66.1

EXAMPLE 4: Observation of the disease and reproduction of the disease under experimental conditions

a) Test No. 1: Observation of the disease

The objective is to take breeding animals at the start of disease and to place them under experimental conditions to follow the progression of the pathology and

describe all the clinical signs thereof. This first test was carried out on 3 breeding pigs aged 10 weeks of which 2 were already ill (suffering from wasting), and on 3 other pigs aged 13 weeks, not having signs of disease. The clinical observation was spread over a period of 37 days. Two pigs of 10 weeks wasted rapidly (pigs 1 and 2, Figure 9) and had to be painlessly killed 5 and 6 days after their arrival. A single pig exhibited hyperthermia over 5 days and diarrhea. Two other pigs exhibited dyspnea and cough, of which one additionally had hyperthermia, greater than 41°C, for the two first days of its stay. Another pig had retarded growth in the second week (pig 6, Figure 9), without any other clinical sign being recorded. On the lesional level, 5 pigs out of 6 exhibited macroscopic lesions of gray pneumonia, the sixth exhibited cicatricial lesions on the lung.

b) Test No. 2: Reproduction of the disease from inocula prepared in farm pigs.

The two sick pigs in test 1 served to prepare inocula which were tested in test 2 on specific-pathogen-free (SPF) pigs. The SPF pigs were aged 9 weeks at the time of inoculation. The clinical and lesional results are shown in Table 5.

Table 5: Summary of the measurements carried out during experimental reproduction of PWD. (The values of the control animals are reported in brackets, the underlined values indicate a difference between infected animals and control animals)

<u>Measurement</u>	<u>Test</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>
<u>Status of the pigs</u>	<u>SPF</u>	<u>SPF</u>	<u>SPF</u>	<u>SPF</u>	<u>SPF</u>	<u>Conventional</u>	<u>Conventional</u>
<u>Age</u>	<u>CNEVA</u>	<u>field</u>	<u>field</u>	<u>CNEVA</u>	<u>CNEVA</u>	<u>Conventional</u>	<u>Conventional</u>
<u>Number</u>	<u>9 weeks</u>	<u>6 weeks</u>	<u>6 weeks</u>	<u>5 weeks</u>	<u>5 weeks</u>	<u>5 weeks</u>	<u>6-7 weeks</u>
<u>Inoculation route</u>	<u>4</u>	<u>6</u>	<u>12</u>	<u>8</u>	<u>8</u>	<u>8</u>	<u>8</u>
	<u>Intratracheal route</u>	<u>Intratracheal route</u>	<u>Intratracheal +</u>	<u>Intratracheal +</u>	<u>Intratracheal +</u>	<u>Intratracheal +</u>	<u>Intratracheal +</u>
			<u>intramuscular route</u>	<u>intramuscular route</u>	<u>intramuscular route</u>	<u>intramuscular route</u>	<u>intramuscular route</u>
<u>Inoculum titer per pig</u>	<u>ND*</u>	<u>ND*</u>	<u>10<sup>4.53</sup> TCID<sub>50</sub> per ml:</u>	<u>10<sup>4.53</sup> TCID<sub>50</sub> per ml:</u>	<u>10<sup>4.53</sup> TCID<sub>50</sub> per ml:</u>	<u>10<sup>4.53</sup> TCID<sub>50</sub> per ml:</u>	<u>10<sup>4.53</sup> TCID<sub>50</sub> per ml:</u>
			<u>1 ml IM + 5 ml IT</u>	<u>1 ml IM + 5 ml IT</u>	<u>1 ml IM + 5 ml IT</u>	<u>1 ml IM + 5 ml IT</u>	<u>1 ml IM + 5 ml IT</u>
<u>Start of hyperthermia</u>	<u>10 days</u>	<u>9-13 days</u>	<u>12-13 days</u>	<u>9-14 days</u>	<u>8-12 days</u>	<u>12 days</u>	<u>12 days</u>
	<u>post-infection</u>	<u>post-infection</u>	<u>post-infection</u>	<u>post-infection</u>	<u>post-infection</u>	<u>post-infection</u>	<u>post-infection</u>
<u>% of pigs in</u>	<u>100%</u>	<u>83%</u>	<u>92%</u>	<u>100%</u>	<u>75%</u>	<u>88%</u>	<u>88%</u>
<u>hyperthermia**</u>							
<u>Number of days of</u>	<u>7</u>	<u>4.5</u>	<u>3.3</u>	<u>5.8</u>	<u>7.5</u>	<u>11.6</u>	<u>11.6</u>
<u>hyperthermia per pig**</u>							
<u>Maximum temperatures</u>	<u>40.4 to 41.7°C</u>	<u>40.6 to 42.3°C</u>	<u>40.2 to 41.6°C</u>	<u>40.3 to 40.8°C</u>	<u>40.6 to 42°C</u>	<u>40.2 to 41.9°C</u>	<u>40.2 to 41.9°C</u>
<u>***</u>							
<u>Hyperthermia****</u>							
<u>% per week</u>							

<u>Measurement</u>	<u>Test</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>
<u>W1</u>		<u>3.5 (3.5)</u>	<u>17 (36)</u>	<u>7 (5)</u>	<u>37 (17)</u>	<u>16 (17)</u>	<u>20 (28)</u>
<u>W2</u>		<u>42 (3.5)</u>	<u>7 (13)</u>	<u>13 (1)</u>	<u>21 (3)</u>	<u>52 (10)</u>	<u>37 (28)</u>
<u>W3</u>		<u>35 (3.5)</u>	<u>33 (10)</u>	<u>28 (7)</u>	<u>62 (2)</u>	<u>34 (12)</u>	<u>79 (17)</u>
<u>W4</u>		<u>21 (3.5)</u>	<u>28 (7)</u>	<u>5 (0)</u>	<u>6 (3)</u>	<u>25 (22)</u>	<u>55 (3)</u>
<u>DMG:</u>							
<u>W1</u>		<u>928 (1053)</u>	<u>417 (357)</u>	<u>564 (620)</u>	<u>650 (589)</u>	<u>401 (407)</u>	<u>509 (512)</u>
<u>W2</u>		<u>678 (1028)</u>	<u>428 (617)</u>	<u>503 (718)</u>	<u>612 (584)</u>	<u>294 (514)</u>	<u>410 (310)</u>
<u>W3</u>		<u>661 (1000)</u>	<u>771 (642)</u>	<u>381 (657)</u>	<u>520 (851)</u>	<u>375 (586)</u>	<u>435 (440)</u>
<u>W4</u>		<u>786 (1100)</u>	<u>550 (657)</u>	<u>764 (778)</u>	<u>641 (696)</u>	<u>473 (610)</u>	<u>451 (681)</u>
<u>Contact pigs</u>		<u>Yes to 100%</u>	<u>Yes to 75%</u>	<u>Not tested</u>	<u>Not tested</u>	<u>Not tested</u>	<u>Not tested</u>
<u>transmission</u>							
<u>% of pulmonary lesions</u>		<u>25</u>	<u>75</u>	<u>0</u>	<u>25</u>	<u>25</u>	<u>12</u>
<u>% of ganglionic lesions</u>		<u>17</u>	<u>33</u>	<u>67</u>	<u>25</u>	<u>50</u>	<u>12</u>

\* ND: not determined,

\*\* hyperthermia when the temperature is greater than 40°C,

\*\*\* range of maximum temperatures recorded at the individual level,

\*\*\*\* the percentage corresponds to the number of temperature recordings greater than 40°C divided by the total number of temperature recordings in the week on all of the pigs.

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In this test, there was no wasting, at the very most a retardation of the growth in the second, third or fourth week after infection. These data illustrate that certain breeding conditions probably favor the expression of the disease.

c) Tests No. 3 to No. 7: Reproduction of the experimental tests

5       The increase in the number of the experimental tests on pigs had the mastering and better characterization of the experimental model as an objective. All of the results are presented in Table 5.

10       Under the experimental conditions, PWD is thus characterized by a long incubation, of 8 to 14 days, true hyperthermia over 2 to 8 days, a decrease in food consumption and a retardation of the increase in weight on the second, third or fourth week post-infection. The lesional table associated with this clinical expression includes, in the main, ganglionic hypertrophy and lesions of pneumonia.

Conclusion

15       The perfection of this experimental model allows the direct etiological role of the PWD circovirus in the disease to be indisputably demonstrated. In addition, this model is an indispensable tool for the understanding of pathogenic mechanisms and the study of future vaccine candidates.

20       EXAMPLE 5: Demonstration of the vaccine composition protective efficacy produced from nucleic fragments of PWD circovirus sequence

1) Animals used for the study

25       Piglets having the PWD disease, reproduced under experimental conditions described in paragraph c) of Example 4, were used in a protocol for evaluating the vaccine composition efficacy, comprising nucleic fragments of PWD circovirus sequence.

2) Tested vaccine composition and vaccination protocol

a) Components used for the study

The plasmids were obtained from the pcDNA3 plasmid of INVITROGENE  
- pcDNA3ORF- plasmids

These plasmids are plasmids which do not carry a PWD circovirus nucleic acid insert and are used as a negative control plasmid.

- pcDNA3ORF1+ plasmid and pcDNA3ORF2+ plasmid

5 The pcDNA3ORF1+ and pcDNA3ORF2+ plasmids are plasmids which carry a nucleic acid insert of the sequence of the PWD circovirus of TYPE B, respectively an insert comprising the nucleic acid fragment SEQ ID No. ~~11~~23 (ORF'1) coding for the Rep protein of sequence SEQ ID No. ~~14~~24 and an insert comprising the nucleic acid fragment SEQ ID No. ~~12~~25 (ORF'2) coding for the protein of sequence SEQ ID No. ~~15~~26, probably corresponding to the capsid  
10 protein, these nucleic constructs comprising the ATG initiation codon of the coding sequence of the corresponding protein.

- GMCSF+ plasmid

GM-CSF (granulocyte/macrophage colony stimulating factor) is a cytokine which occurs in the development, the maturation and the activation of macrophages,  
15 granulocytes and dendritic cells which present an antigen. The beneficial contribution of the GM-CSF in vaccination is considered to be a cellular activation with, especially, the recruitment and the differentiation of cells which present an antigen.

This pcDNA3-GMCSF+ plasmid carries a nucleic acid insert coding for the  
20 granulocyte/macrophage colony stimulation factor, the GM-CSF protein.

The gene coding for this GM-CSF protein was cloned and sequenced by Inumaru et al. (Immunol. Cell Biol., 1995, 73 (5), 474-476). The pcDNA3-GMCSF+ plasmid was obtained by Dr. B. Charley of INRA of Jouy-en-Josas (78, France).

25 - Recombinant baculoviruses

The so-called ORF- baculoviruses are viruses not carrying any insert comprising a nucleic acid fragment capable of expressing a PWD circovirus protein.

The so-called ORF1+ (BAC ORF1+) or ORF2+ (BAC ORF2+) baculoviruses are recombinant baculoviruses respectively carrying an insert

comprising a nucleic acid fragment SEQ ID No. ±±23 (ORF'1) and an insert comprising the nucleic acid fragment SEQ ID No. ±225 (ORF'2).

- Adjuvant

5 The adjuvant supplied by the Seppic Company, a subsidiary of AIR LIQUIDE, is the adjuvant corresponding to the reference AIF SEPPIC.

b) Vaccination protocol

Weaned piglets aged 3 weeks are divided into four batches A, B, C and D each comprising 8 piglets.

10 Batches A, B and C, aged 3 weeks, each receive a first injection (injection M1) of 1 ml containing 200 micrograms of plasmids (naked DNA) in PBS, pH: 7.2, by the intramuscular route for each of the plasmids mentioned below for each batch, then, at the age of 5 weeks, a second injection (injection M2) comprising these same plasmids. A third injection is carried out simultaneously on the other side of the neck. This third injection comprises 1 ml of a suspension containing  $5 \cdot 10^6$  cells  
15 infected by recombinant baculoviruses and 1 ml of AIF SEPPIC adjuvant.

Batch A (F1) (control batch):

- first injection

pcDNA3ORF1- plasmid, pcDNA3ORF2- plasmid and GMCSF+ plasmid.

- second and third injection (simultaneous)

20 pcDNA3ORF1- plasmid, pcDNA3ORF2- plasmid and GMCSF+ plasmid;

Cells transformed by baculoviruses not containing any nucleic acid insert coding for a PWD circovirus protein;

AIF SEPPIC adjuvant.

25 Batch B (F2) (control batch):

- first injection

pcDNA3ORF1- plasmid, pcDNA3ORF2- plasmid and GMCSF+ plasmid;

- second and third injection (simultaneous)

pcDNA3ORF1- plasmid, pcDNA3ORF2- plasmid and GMCSF+ plasmid;

Cells transformed by baculoviruses not containing any nucleic acid insert coding for a PWD circovirus protein;

5 AIF SEPPIC adjuvant.

Batch C (F3):

- first injection

pcDNA3ORF1+ plasmid, pcDNA3ORF2+ plasmid and GMCSF+ plasmid;

10 - second and third injection (simultaneous)

pcDNA3ORF1+ plasmid, pcDNA3ORF2+ plasmid and GMCSF+ plasmid;

15 Cells transformed by BAC ORF1+ and BAC ORF2+ recombinant baculoviruses capable of respectively expressing the Rep protein of sequence SEQ ID No. 1424 and the protein of sequence SEQ ID No. 1526 of the PWD circovirus of TYPE B.

Batch D (F4) (control batch): no injection

The batches of piglets B, C and D are infected (tested) at the age of 6 weeks although batch A is not subjected to the test.

20 3) Observation of the batches

- counting of coughing/sneezing: 15 minutes/batch/day;

- consistency of fecal matter: every day;

- regular recordings: weekly taking of blood, weighing;

- weighing of food refuse: 3 times per week;

25 - calculation of the daily mean gain in weight (dmg);

The daily mean gains were calculated for each of the batches over a period of 28 days following testing (cf. Figure 10), an intermediate calculation of the dmg was likewise carried out for each of the batches over the first and second periods of 14 days. The results obtained are reported below in Table 6.

Table 6: Daily mean gains

	F1	F2	F3	F4
d0-d14	411 g	450 g	511 g	461 g
d14-d28	623 g	362 g	601 g	443 g
d0-d28	554 g	406 g	556 g	452 g

## - Measurement of hyperthermia

- 5 The measurement of hyperthermia, of greater than 41°C (cf. Figure 11) and greater than 40.2°C, was carried out for each of the batches over a total period of 28 days following testing. The results obtained, corresponding to the ratio expressed as a percentage between the number of recordings of heat of greater than 41°C (or greater than 40.2°C) and the total number of recordings of heat carried out on all of
- 10 the pigs per one-week period are reported below in Tables 7 and 8, respectively for the hyperthermia measurements of greater than 41°C and greater than 40.2°C.

Table 7: Hyperthermia > 41°C

	F1	F2	F3	F4
W1	4.1	0.	0.	0.
W2	10.7	16.	0.	8.9
W3	4.7	27.	0.	45.
W4	0.	0.	0.	7.5

Table 8: Hyperthermia > 40.2

	F1	F2	F3	F4
W1	29.1	10.41	29.1	20.8
W2	28.5	39.2	10.7	37.5
W3	14.3	68.7	25.0	81.2
W4	3.3	17.5	20.0	55

#### 4) Conclusion

5 The recordings carried out clearly show that the animals which received the three injections of a vaccine composition comprising nucleic acid fragments of PWD circovirus according to the invention and/or capable of expressing recombinant proteins of PWD circovirus, in particular of type B, did not exhibit hyperthermia (cf. Figure 10). These animals additionally did not experience a decline in their growth, the dmgs being comparable to those of uninfected control animals (cf. 10 Figure 9). They did not exhibit any particular clinical sign.

These results demonstrate the efficacious protection of the piglets against infection with a PWD circovirus of the invention, the primary agent responsible for PWD or FPW, provided by a vaccine composition prepared from a nucleic acid fragment of the nucleic sequence of PWD circovirus according to the invention, in 15 particular of type B, and/or from recombinant proteins encoded by these nucleic acid fragments.

These results in particular show that the proteins encoded by the ORF1 and ORF2 of PWD circovirus according to the invention are immunogenic proteins inducing an efficacious protective response for the prevention of infection by a 20 PWD circovirus.

EXAMPLE 6: Serological diagnosis of PWD circovirus by immunodetermination using recombinant proteins or synthetic peptides of PWD circovirus

A - Serological diagnosis with recombinant proteins

5 The identification and the sequencing of porcine PWD circovirus allow recombinant proteins of PWD circovirus to be produced by the techniques of genetic recombination well known to the person skilled in the art.

By these techniques, recombinant proteins encoded, in particular, by the ORF'2 of the PWD circovirus, type B, were expressed by transformed Sf9 insect cells and then isolated.

10 These recombinant proteins encoded by the ORF'2 are extracted, after culture of the transformed Sf9 cells, by thermal cell lysis by means of 3 cycles of freezing/thawing to -70°C/+37°C. Healthy Sf9 cells or nontransformed control Sf9 cells are also lyzed.

15 These two antigenic fractions originating from nontransformed control Sf9 cells and Sf9 cells expressing the ORF'2 are precipitated at 4°C by a 60% plus or minus 5% saturated ammonium sulfate solution. Determination of total proteins is carried out with the aid of the Biorad kit. 500 ng of control Sf9 proteins and of semipurified Sf9 proteins expressing the ORF'2, in solution in 0.05 M bicarbonate buffer pH 9.6, are passively adsorbed at the bottom of 3 different cupules of a Nunc  
20 Maxisorp microplate by incubation for one night at +4°C.

The reactivity of pig sera with respect to each of these antigenic fractions is evaluated by an indirect ELISA reaction of which the experimental protocol is detailed below:

- Saturation step: 200 µl/cupule of PBS1X/3% semi-skimmed milk, 1 h 30  
25 incubation at 37°C.
- Washing: 200 µl/cupule of PBS1X/Tween 20: 0.05%, 3 rapid washes.
- Serum incubation step: 100 µl/cupule of serum diluted to 1/100 in PBS1X/semi-skimmed milk, 1%/Tween 20: 0.05%, 1 h incubation at 37°C.



- Washing: 200  $\mu$ l/cupule of PBS1X/Tween 20: 0.05%, 2 rapid washes followed by 2 washes of 5 min.

- Conjugate incubation step: 50  $\mu$ l/cupule of rabbit anti-pig conjugate diluted to 1/1000 in PBS1X/semi-skimmed milk, 1%/Tween 20: 0.05%, 1 h incubation at 37°C.

- Washing: 200  $\mu$ l/cupule of PBS1X/Tween 20: 0.05%, 2 rapid washes followed by 2 washes of 5 min.

- Visualization step: 100  $\mu$ l/cupule of OPD substrate/citrate buffer/H<sub>2</sub>O<sub>2</sub>, 15 min incubation at 37°C.

- Stopping of reaction: 50  $\mu$ l/cupule of 1 N H<sub>2</sub>SO<sub>4</sub>.

- Reading in a spectrophotometer at 490 nm.

### Results

The results obtained are shown below in Table 9.

Table 9

Antigens	Reactivity of Pig Serum not inoculated with Circovirus	Reactivity of Pig Serum inoculated with Circovirus
Purified Sf9 control	0.076	0.088
Sf9 expressing purified ORF'2	0.071	1.035

The results are expressed in optical density measured in a spectrophotometer at 490 nm during analysis by ELISA of the reactivity of pig sera which are or are not inoculated with the type B PWD circovirus according to the protocol indicated above.

### B - Serological Diagnosis by Synthetic Peptide

The epitopic mapping of the proteins encoded, for example, by the nucleic sequences ORF1 and ORF2 of the two types of PWD circovirus (types A and B) additionally allowed immunogenic circoviral epitopes to be identified on the proteins encoded by the nucleic sequences ORF'1 and ORF'2 as well as the specific

5 epitopes of the protein encoded by the nucleic sequence ORF'2 of the type B PWD circovirus. Four specific epitopes of the type B PWD circovirus and one epitope common to the two types of PWD circovirus situated on the protein encoded by the nucleic sequence ORF'2 were synthesized in peptide form. The equivalent peptides in the circovirus of type A were likewise synthesized. All these peptides were evaluated as diagnostic antigens within the context of carrying out a serological test.

#### Results

The results obtained are shown in Table 10 below.

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Table 10: Results of the evaluation as a diagnostic antigen of synthetic peptides encoded by the nucleic sequences ORF2 and ORF'2 of PWD circovirus of type A and B.

Infected pig serum reactivity						
Circovirus B						
Peptide	Type	Position	AA sequence	SPF	Conventional 1	Conventional 2
de	PWD circovirus			D0/D54	D0/D42	D0/D42
SEQ ID NO: 29	121	71-85	VDMRMRFNINDFLPPG	+/-, +++	+/-, +++	-, +++
SEQ ID NO: 55	177	70-84	NVNELREFNIGQFLPP	+/-, +	+/-, +/-	+/-, -
SEQ ID NO: 30	131	115-129	QGDRGVGSSAVILDD	+/-, +/-	++, ++	+/-, +
SEQ ID NO: 56	188	114-127	TSNQRGVGSTVVIL	+/-, -	-, +/-	+/-, +/-
SEQ ID NO: 31	133	119-134	GVGSSAVILDDNVFTK	-, ++	++, ++	+/-, ++
SEQ ID NO: 57	189	118-132	RGVGSTVVILDANFY	+/-, -	-, +/-	+/-, +/-
SEQ ID NO: 58	146	171-185	FTIDYFQPNKRNQL	-, +/-	-, ++	-, ++
SEQ ID NO: 59	202	170-184	DQTIDWFQPNKRNQ	++, +	+/-, ++	++, ++
				+++		
SEQ ID NO: 32	152	195-209	VDHVGLGTAFENSIY	-, ++	+++	+/-, +
SEQ ID NO: 60	208	194-208	NVEHTGLGYALQNAT	-, -	-, -	-, -

+/-, +, +, +, +, +, +, +, +. Increasing intensities of the reactivities observed in Spot peptides on a nitrocellulose membrane. The porcine sera tested are from animals experimentally infected with the circovirus of type B within the animal houses of the CNEVA. Samples are taken from the animals before inoculation on d0 and 42 days or 54 days after inoculation, on d42, d54.

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EXAMPLE 7: Characterization of the specific epitopes of the PWD circovirus of type B

The proteins encoded by the ORF2 of the porcine circoviruses of type A and B were chosen for this study. For each of the ORF2s (types A and B), 56 peptides of 15 amino acids which overlap every 4 amino acids were synthesized, thus covering the whole of the protein (cf. Table 11 below).

Table 11: Sequence of amino acids of the 56 peptides of 15 amino acids synthesized from the nucleic sequence ORF<sup>1</sup><sub>2</sub> (type B) and ORF2 (type A) of PWD circovirus with their corresponding spot number (cf. Figure 12)

Type B ORF <sup>1</sup> <sub>2</sub> Spot No.			Type A ORF2Spot No.		
	Spot No.	Sequence		Spot No.	Sequence
SEQ ID NO:61	107	HRPRSHLGOILRRRP	SEQ ID NO:84	163	TRPRSHLGNILRRRP
SEQ ID NO:62	108	SHLGOILRRRPWL VH	SEQ ID NO:85	164	SHLGNILRRRPYL VH
SEQ ID NO:63	109	QILRRRPWL VHPRHR	SEQ ID NO:86	165	NILRRRPYL VHPAFR
SEQ ID NO:64	110	RRPWL VHPRHRYRWR	SEQ ID NO:87	166	RRPYL VHPAFRNRYR
SEQ ID NO:65	111	LVHPRHRYRWRRKNG	SEQ ID NO:88	167	LVHPAFRNRYRWRRK
SEQ ID NO:66	112	RHRYRWRRKNGIENT	SEQ ID NO:89	168	AFRNRYRWRRKTGIF
SEQ ID NO:67	113	RWRRKNGIENTRLSR	SEQ ID NO:90	169	RYRWRRKTGIFNSRL
SEQ ID NO:68	114	KNGIENTRLSRTEGY	SEQ ID NO:91	170	RRKTGIFNSRLSREF
SEQ ID NO:69	115	FNTRL SRTEGYTVKR	SEQ ID NO:92	171	GIFNSRLSREFVLT I
SEQ ID NO:70	116	LSRTEGYTVKR TTVR	SEQ ID NO:93	172	SRLSREFVLTIRGGH
SEQ ID NO:71	117	FGYTVKR TTVRTPSW	SEQ ID NO:94	173	REFVLTIRGGHSOPS
SEQ ID NO:72	118	VKR TTVRTPSWAVDM	SEQ ID NO:95	174	LTIRGGHSOPSWNVN
SEQ ID NO:73	119	TVRTPSWAVDMMRFN	SEQ ID NO:96	175	GGHSOPSWNVNELRF
SEQ ID NO:74	120	PSWAVDMMRFNINDF	SEQ ID NO:97	176	OPSWNVNELRFNIGO
SEQ ID NO:29	121	VDMRFNINDFLPPG	SEQ ID NO:98	177	NVNELRFNIGOQLPP
SEQ ID NO:75	122	RFNINDFLPPGGGSN	SEQ ID NO:99	178	LRFNIGOQLPPSGGT
SEQ ID NO:76	123	NDFLPPGGGSNPRSV	SEQ ID NO:100	179	IGQLPPSGGTNPLP
SEQ ID NO:77	124	PPGGGSNPRSVPF EY	SEQ ID NO:101	180	LPPSGGTNPLPLPFQ
SEQ ID NO:78	125	GSNPRSVPF EYRIR	SEQ ID NO:102	181	GGTNPLPLPFQYYRI
SEQ ID NO:79	126	RSVPF EYRIRKVKV	SEQ ID NO:103	182	PLPLPFQYYRIRKAK
SEQ ID NO:80	127	FEYYRIRKVKVEFWP	SEQ ID NO:104	183	PFQYYRIRKAKYEFY
SEQ ID NO:81	128	RIRKVKVEFWPCSPI	SEQ ID NO:105	184	YRIRKAKYEFYPRDP
SEQ ID NO:82	129	VKVEFWPCSPITQGD	SEQ ID NO:106	185	KAKYEFYPRDPITSN
SEQ ID NO:83	130	FWPCSPITQDGRGVG	SEQ ID NO:107	186	EFYPRDPITSNORGV
SEQ ID NO:30	131	SPITQDGRGVGSSAV	SEQ ID NO:108	187	RDPIITSNORGVGSTV
SEQ ID NO:31	132	QGDRGVGSSAVILDD	SEQ ID NO:109	188	TSNORGVGSTVVILD
SEQ ID NO:110	133	GVGSSAVILDDNFVT	SEQ ID NO:136	189	RGVGSTVVILDANFV
SEQ ID NO:111	134	SAVILDDNFVTKATA	SEQ ID NO:137	190	STVVILDANFVTPST
SEQ ID NO:112	135	LDDNFVTKATALTYD	SEQ ID NO:138	191	ILDANFVTPSTNLAY
SEQ ID NO:113	136	FVTKATALTYDPYVN	SEQ ID NO:139	192	NEVTPSTNLAYDPYI
SEQ ID NO:114	137	ATALTYDPYVNYSSR	SEQ ID NO:140	193	PSTNLAYDPYINYSS
SEQ ID NO:115	138	TYDPYVNYSSRIITIT	SEQ ID NO:141	194	LAYDPYINYSSRHTI

Type B ORF1 Spot No.			Type A ORF2 Spot No.		
Spot No.	Sequence		Spot No.	Sequence	
SEQ ID NO:116	139	YVNYSSRHTITOPES	SEQ ID NO:142	195	PYINYSSRHTIROPE
SEQ ID NO:117	140	SSRHTITOPESYHSR	SEQ ID NO:143	196	YSSRIITROPFTYHS
SEQ ID NO:118	141	TITOPFSYHSRYFTP	SEQ ID NO:144	197	HTIROPFTYHSRYFT
SEQ ID NO:119	142	PFSYHSRYFTP KPVL	SEQ ID NO:145	198	QPFTYHSRYFTP KPPE
SEQ ID NO:120	143	HSRYFTP KPVL DFTI	SEQ ID NO:146	199	YHSRYFTP KPPEL DOT
SEQ ID NO:121	144	FTP KPVL DFTIDYYFO	SEQ ID NO:147	200	YFTP KPPEL DOTIDWF
SEQ ID NO:122	145	PVL DFTIDYFOPNNK	SEQ ID NO:148	201	KPEL DOTIDWFOPNN
SEQ ID NO:123	146	FTIDYFOPNNKRNOL	SEQ ID NO:149	202	DOTIDWFOPNNKRNOL
SEQ ID NO:124	147	YFOPNNKRNOLWLRL	SEQ ID NO:150	203	DWFOPNNKRNOLWLH
SEQ ID NO:125	148	NNKRNOLWLRLQTAG	SEQ ID NO:151	204	PNNKRNOLWLHLNTH
SEQ ID NO:126	149	NOLWLRLQTAGNV DH	SEQ ID NO:152	205	RNOLWLHLNTHTNVE
SEQ ID NO:127	150	LRLQTAGNV DHVGLG	SEQ ID NO:153	206	WLHLNTHTNVEHTGL
SEQ ID NO:128	151	TAGNV DHVGLGTAFE	SEQ ID NO:154	207	NTHTNVEHTGLGYAL
SEQ ID NO:32	152	VDHVGLGTAFENSIY	SEQ ID NO:155	208	NVEHTGLGYALONAT
SEQ ID NO:129	153	GLGTAFENSIYDOEY	SEQ ID NO:156	209	TGLGYALONATTAON
SEQ ID NO:130	154	AFENSIYDOEYNIRV	SEQ ID NO:157	210	YALONATTAQNYVVR
SEQ ID NO:131	155	SIYDOEYNIRVTMYV	SEQ ID NO:158	211	NATTAQNYVVRLTIY
SEQ ID NO:132	156	OEYNIRVTMYVQFRE	SEQ ID NO:159	212	AQNYVVRLTIYVQFER
SEQ ID NO:133	157	IRVTMYVQFREFNFK	SEQ ID NO:160	213	VVRLTIYVQFREFIL
SEQ ID NO:134	158	MYVQFREFNFKDPPL	SEQ ID NO:161	214	TIYVQFREFILKDPPL
SEQ ID NO:135	159	VQFREFNFKDPPLNP	SEQ ID NO:162	215	YVQFREFILKDPPLNE

These peptides were synthesized according to the "spot" method which consists in simultaneous synthesis of a large number of peptides on a cellulose solid support, each site of synthesis of a peptide constituting a spot (Synt:em, NIMES). This method involves orientation of the peptides on the plate, these being fixed covalently by the carboxy-terminal end. A spot represents approximately 50 nmol of peptide.

The reference of the spots and corresponding peptide sequences is given in Table 11.

These membranes were used for immunoreactivity tests with respect to serum of SPF pigs which were or were not infected experimentally with the type B PWD circoviral strain as well as with respect to sera of infected pigs from conventional farms (conventional farms 1 or 2). This study allowed specific immunoreactive peptides of the circovirus of type B corresponding to the spots No. 121, No. 132, No. 133 and No. 152 (respectively of amino acid sequences SEQ ID No. 177, 29, SEQ ID No. 187, 30, SEQ ID No. 193, 1 and SEQ ID No. 203, 2) to be demonstrated. An illustration is shown in Figure 12 where the membranes are visualized with an infected pig serum coming from a conventional farm. Nonspecific



immunoreactive peptides of type [lacuna] were likewise demonstrated, among which we shall keep the peptide No. 146 SEQ ID No. 123 which is strongly immunogenic.

5 A comparison between the peptide sequences of circoviruses of type A and B (Figure 13) indicates a divergence ranging from 20 to 60% for the specific immunoreactive peptides of the type B, and a weaker divergence (13%) between the nonspecific peptides.

10 EXAMPLE 8: Protection of Swine From Post-Weaning Multisystemic Wasting Syndrome (PMWS) Conferred by Procine Circovirus TypeB (PCV-B) ORF'2 Protein

The ORF'1-encoded protein (REP) and ORF'2-encoded putative capsid protein of PCV-B were expressed, either in insect cells by recombinant baculovirus vectors, or in mammalian cell lines by transfection with plasmidic expression vectors. These two circovirus-derived proteins were detectable in both expression system. As evaluated by weight gains, hyperthermia and absence of lesions following challenge, the pigs were protected against a virulent circovirus challenge after one first DNA immunization with plasmids directing ORF'2 protein and GM-CSF expression and a second injection, 15 days later, with the same plasmid preparation plus the ORF'2 recombinant protein. A lower level of protection was observed when the pigs were vaccinated with ORF'1 protein, as opposed to pigs vaccinated with ORF'2 protein.

A. Development of an experimental model of PMWS in swine:

Eight 3 week-old SPF pigs were inoculated intratracheally (5 ml) and intramuscularly (1 ml).

25 B. Production and control of PCV-B plasmids:

PCV-B ORF'1 and ORF'2 genes, isolated from PCV-B challenge strain, have been cloned into vector plasmid pcDNA3.1.

All constructs have been validated through a partial sequencing of the PCV-B genes in the final plasmids and expression control by immunoperoxidase on PK15 cells respectively transfected with each plasmid, using swine polyclonal antibodies.

Plasmid encoding GM-CSF has been co-administred.

C. Construction of recombinant baculoviruses:

ORF'1 and ORF'2 proteins were expressed under polyhedrin promoter control. Recombinant proteins were detected by western-blot using swine polyclonal antibodies.

5

D. Vaccination and challenge:

Four groups of 7 pigs were vaccinated intramuscularly at day 0 (Do), two weeks later, they received the same plasmid preparation plus the recombinant baculovirus.

10

E. Monitoring:

All groups of pigs were housed in isolated experimental units with air filtration and low air pressure. Clinical observations and rectal temperatures were recorded every day. The pigs were weighed weekly.

F. Conclusions

15

Expression of PCV-B ORF'2 or PCV-B ORF'1 in swine resulted in a significantly enhanced level of protection as evaluated by weight evolution and body temperature evolution following challenge with PCV-B circovirus. These results are summarized in Figures 14 and 15.

20

The invention described herein may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The specific embodiments previously described are therefore to be considered as illustrative of, and not limiting, the scope of the invention. Additionally, the disclosure of all publications and patent applications cited above and below, including International Patent Application No. PCT/FR98/02634, filed December 4, 1998, and published as International Publication No. WO 99/29871 on June 17, 1999, are expressly incorporated herein by reference in their entireties to the same extent as if each were incorporated by reference individually.

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**Bibliographic references**

- Allan, G.M. et al., 1995, Vet. Microbiol., 44: 49-64.
- Barany, F., 1911, PNAS. USA, 88: 189-193.
- Boulton, L.H. et al., 1997, J. Gen. Virol., 78 (Pt 6), 1265-1270.
- Buckholz, R.G., 1993, Yeast systems for the expression of heterologous gene products. Curr. Op. Biotechnology 4: 538-542.
- Burg, J.L. et al., 1996, Mol. and Cell. Probes, 10: 257-271.
- Chu, B.C.F. et al., 1986, NAR, 14: 5591-5603.
- Chu, P.W.G. et al., 1993, Virus Research, 27: 161-171.
- Clark, E.G., 1997, American Association of Swine Practitioners, 499-501.
- Daft, B. et al., 1996, American Association of Veterinary Laboratory Diagnosticians, 32.
- Derse, D. et al., 1995, J. Virol., 69(3): 1907-1912.
- Duck, P. et al., 1990, Biotechniques, 9: 142-147.
- Dulac, G.C. et al., 1989, Can. J. Vet. Res., 53: 431-433.
- Edwards, C.P., and Aruffo, A., 1993, Current applications of COS cell based transient expression systems. Curr. Op. Biotechnology 4: 558-563.
- Edwards, S. et al., 1994, Vet. Rec., 134: 680-681.
- Erlich, H.A., 1989, In PCR Technology. Principles and Applications for DNA Amplification. New York: Stockton Press.
- Felgner, et al., 1987, Proc. Natl. Acad. Sci., 84: 7413.
- Fontes, E.P.B. et al., 1994, J. Biol. Chem., Vol. 269, No. 11: 8459-8465.
- Fraley et al., 1980, J. Biol. Chem., 255: 10431.
- Guateli, J.C. et al., 1990, PNAS. USA, 87: 1874-1878.
- Hackland, A.F. et al., 1994, Arch. Virol., 139: 1-22.
- Hanson, S.F. et al., 1995, Virology, 211: 1-9.
- Harding, J.C., 1997, American Association of Swine Practitioners, 503.
- Harding, R.M. et al., 1993, Journal of General Virology, 74: 323-328.

- Harding, J.C. and Clark, E.G., 1997, Swine Health and Production, Vol. 5, No. 5: 201-203.
- Heyraud-Nitschke, F. et al., 1995, Nucleic Acids Research, Vol. 23, No. 6.
- Horner, G.W., 1991, Surveillance 18(5): 23.
- Houben-Weyl, 1974, in Methode der Organischen Chemie, E. Wunsch Ed., Volume 15-I and 15-II, Thieme, Stuttgart.
- Huygen, K. et al., 1996, Nature Medicine, 2(8): 893-898.
- Innis, M.A. et al., 1990, in PCR Protocols. A guide to Methods and Applications, San Diego, Academic Press.
- Kaneda, et al., 1989, Science, 243: 375.
- Kievitis, T. et al., 1991, J. Virol. Methods, 35: 273-286.
- Kohler, G. et al., 1975, Nature, 256(5517): 495-497.
- Kwoh, D.Y. et al., 1989, PNAS. USA, 86: 1173-1177.
- Ladany, S. et al., 1989, J. Clin. Microbiol. 27: 2778-2783.
- Lazarowitz, S.G. et al., 1989, The EMBO Journal, Vol. 8 No. 4: 1023-1032.
- Luckow, V.A., 1993, Baculovirus systems for the expression of human gene products. Curr. Op. Biotechnology 4: 564-572.
- Mankertz, A. et al., 1997, J. Virol., 71: 2562-2566.
- Matthews, J.A. et al., 1988, Anal. Biochem., 169: 1-25.
- McNeilly, F. et al., 1996, Vet. Immunol. Immunopathol., 49: 295-306.
- Meehan, B.M. et al., 1997, J. Gen. Virol. 78: 221-227.
- Merrifield, R.D., 1966, J. Am. Chem. Soc., 88(21): 5051-5052.
- Midoux, 1993, Nucleic Acids Research, 21: 871-878.
- Miele, E.A. et al., 1983, J. Mol. Biol., 171: 281-295.
- Murphy, F.A. et al., 1995, Sixth Report of the International Committee on Taxonomy of Viruses. Springer-Verlag Wien New York.
- Nayar, G.P. et al., 1997, Can. Vet. J. 38(6): 385-386.
- Olins, P.O., and Lee, S.C., 1993, Recent advances in heterologous gene expression in E.coli. Curr. Op. Biotechnology 4: 520-525.

Pagano et al., 1967, J. Virol., 1: 891.

Rolfs, A. et al., 1991, In PCR Topics. Usage of Polymerase Chain reaction in Genetic and Infectious Disease. Berlin: Springer-Verlag.

Sambrook, J. et al., 1989, In Molecular cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanchez-Pescador, R., 1988, J. Clin. Microbiol., 26(10): 1934-1938.

Segev D., 1992, in "Non-radioactive Labeling and Detection of Biomolecules". Kessler C. Springer Verlag, Berlin, New-York: 197-205.

Shiver, J.W., 1995, in Vaccines 1995, eds Chanock, R.M. Brown, F. Ginsberg, H.S. & Norrby, E., pp. 95-98, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Tascon, R.E. et al., 1996, Nature Medicine, 2(8): 888-892.

Tischer, I. et al., 1982, Nature, 295: 64-66.

Tischer, I. et al., 1986, Arch. Virol., 91: 271-276.

Tischer, I. et al., 1988, Zentralbl Bakteriol Mikrobiol Hyg [A] 270: 280-287.

Tischer, I. et al., 1995, Arch. Virol., 140: 737-743.

Urdea, M.S., 1988, Nucleic Acids Research, II: 4937-4957.

Walker, G.T. et al., 1992, NAR 20: 1691-1696.

Walker, G.T. et al., 1992, PNAS. USA, 89: 392-396.

White, B.A. et al., 1997, Methods in Molecular Biology, 67, Humana Press, Towota.

Zhao, T.M. et al., 1996, Proc. Natl. Acad. Sci., USA 93(13): 6653-6648.



~~WO 99/29871~~

PCT/FR98/02634

~~-90-~~ Attorney Docket No. 65691/176

**CLAIMS**

We Claim:

1. ~~Nucleotide~~1. A vaccine comprising a nucleotide sequence of the genome of ~~PWD~~Porcine circovirus selected from the sequences SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 9, SEQ ID No. 10 type B, or one of their fragments a homologue or fragment thereof, and an acceptable pharmaceutical or veterinary vehicle.

~~2.~~

~~Nucleotide sequence of PWD circovirus, characterized in that it is selected from:~~

~~a) a nucleotide sequence of a specific fragment of a sequence~~2. A vaccine according to Claim 1;

~~b) a claim 1, wherein the nucleotide sequence homologous to a nucleotide sequence such as defined in a);~~

5 ~~c) a nucleotide sequence complementary to a nucleotide sequence such as defined in a) or b), and a nucleotide sequence of their corresponding RNA;~~

~~d) a nucleotide sequence capable of hybridizing under stringent conditions with a sequence such as defined in a), b) or c);~~

10 ~~e) a nucleotide sequence comprising a sequence such as defined in a), b), c) or d); and~~

~~f) a nucleotide sequence modified by a nucleotide sequence such as defined in a), b), c), d) or e).~~

15 ~~3. Nucleotide sequence according to Claim 2, characterized in that it is selected from the sequences SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13 or one of their fragments.~~  
sequence is selected from SEQ ID No. 15 or SEQ ID No. 19.

20

3. A vaccine according to claim 1, wherein the homologue has at least 80% sequence identity to SEQ ID No. 15 or SEQ ID No. 19.

4. Nucleotide A sequence vaccine according to ~~Claim 2,~~  
~~characterized in that it comprises a~~ claim 1, wherein the nucleotide  
sequence is selected from: SEQ ID No. 23 or SEQ ID No. 25, or a homologue or  
fragment thereof.

- 5 a) ~~a nucleotide sequence according to Claim 3;~~  
b) ~~a nucleotide sequence of a specific fragment of a~~  
~~sequence such as defined in a);~~  
c) ~~a homologous nucleotide sequence having at least 80%~~  
10 ~~identity with a nucleotide sequence such as defined~~  
~~in a) or b);~~  
d) ~~a complementary nucleotide sequence or sequence of~~  
~~RNA corresponding to a sequence such as defined in~~  
~~a), b) or c); and~~  
15 e) ~~a nucleotide sequence modified by a sequence such as~~  
~~defined in a), b), c) or d).~~

5. Nucleotide A sequence vaccine according to ~~one of Claims 2~~  
~~to~~ claim 4, characterized in that wherein the specific fragment  
20 nucleotide homologue has at least 80% sequence ~~comprises a~~  
~~nucleotide sequence selected from the following~~  
~~sequences:~~ identity to SEQ ID No. 23 or SEQ ID No. 25.

- a) ~~5' TGTGGCGA 3';~~  
b) ~~5' AGTTTCCT 3';~~  
25 c) ~~5' TCATTTAGAGGCTCTTTCAG 3';~~  
d) ~~5' GTCACCT 3';~~  
e) ~~5' GTGGTTGC 3';~~  
f) ~~5' AGCCCAGG 3';~~  
g) ~~5' TTGGCTGC 3';~~  
30 h) ~~5' TCTAGCTCTGGT 3';~~  
i) ~~5' ATCTCAGCTCGT 3';~~  
j) ~~5' TGTCTCTCTT 3';~~  
k) ~~5' TCTCTAGA 3';~~  
l) ~~5' TGTACCAA 3';~~

~~m) 5' TCCGTCTT 3';~~

~~and their complementary sequences.~~

5 ~~6. Polypeptide encoded by a~~6. A vaccine according to claim 4,  
wherein the nucleotide sequence according to one of Claims 1 to 5 is  
SEQ ID No. 25.

~~7.~~

10 ~~Polypeptide according to Claim 6, characterized in~~  
~~that its sequence is represented by a specific fragment~~  
~~of one of the six sequences of amino acids shown in~~  
~~Figure 27. A vaccine comprising a polypeptide encoded by a nucleotide~~  
~~sequence of the genome of PCVB, or in Figure 8, a homologue or fragment~~  
~~thereof, and an acceptable pharmaceutical or veterinary vehicle.~~

15 ~~8. Polypeptide~~8. A vaccine according to Claim 6 or claim 7,  
~~characterized in that it is selected from~~wherein the  
~~polypeptides of sequences~~homologue has at least 80% sequence identity  
~~to SEQ ID No. 6, 15 or SEQ ID No. 7, SEQ ID No. 8, SEQ ID No.~~  
20 ~~14, SEQ ID No. 15, SEQ ID No. 16 or one of their~~  
~~fragments.~~19.

~~9.~~

25 ~~Polypeptide characterized in that it comprises a~~  
~~polypeptide selected from:~~

~~a) a specific fragment of at least 5 amino acids of a~~  
~~polypeptide~~9. A vaccine according to one of Claims 6  
~~to 8;~~

30 ~~b) a polypeptide homologous to a polypeptide such as~~  
~~defined in a);~~

~~c) a specific biologically active fragment of a~~  
~~polypeptide such as defined in a)~~claim 7, wherein the  
nucleotide sequence is selected from SEQ ID No. 23 or SEQ ID No. 25, or  
~~b); and~~

d) ~~a polypeptide modified by a polypeptide such as defined in a), b) a homologue or c).~~

10. ~~Polypeptide according to Claim 9, characterized in that it comprises a polypeptide selected from the polypeptides of sequences SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19 and SEQ ID No. 20. fragment thereof.~~

10. A vaccine according to claim 9, wherein the homologue has at least 80% sequence identity to SEQ ID No. 23 or SEQ ID No. 25.

~~11. Nucleotide sequence coding for a polypeptide 11.~~

A vaccine according to Claims 7 to 10. claim 9, wherein the nucleotide sequence is SEQ ID No. 25.

~~12.~~

~~Nucleotide sequence utilizable as a primer or probe, characterized in that said sequence is selected from the nucleotide sequences according to one of Claims 1 to 5 and 11.~~ 12. A vaccine according to claim 7, wherein the polypeptide has the amino acid sequence of SEQ ID No. 24 or SEQ ID No. 26.

~~13. Nucleotide A sequence vaccine according to Claim claim 12, characterized in that said wherein the polypeptide has the amino acid sequence is one of the primer of the pairs of primers selected from the following pairs: SEQ ID No. 26.~~

~~a)~~

~~5' GTG TGC TCG ACA TTG GTG TG 3', and~~

~~5' TGG AAT GTT AAC GAG CTC AG 3';~~

~~b) 5' GTG TGC TCG ACA TTG GTG TG 3', and~~

~~5' CTC GCA GCC ATC TTG GAA TG 3';~~

~~c) 5' CGC GCG TAA TAC GAC TCA CT 3', and~~

~~5' GTG TGC TCG ACA TTG GTG TG 3';~~

~~d) 5' CGC GCG TAA TAC GAC TCA CT 3', and~~

~~5' CTC GCA GCC ATC TTG GAA TG 3',~~

e) ~~5' CCT GTC TAC TGC TGT GAG TAC CTT GT 3', and~~

~~5' GCA GTA GAC AGG TCA CTC CGT TGT CC 3'.~~

14. ~~Nucleotide sequence 14. A vaccine according to Claim 12, characterized in that said sequence is a specific consensus sequence claim 7, wherein the homologue has at least 80% sequence identity to SEQ ID No. 24 or SEQ ID No. 26.~~

15. ~~A vaccine according to claim 14, wherein the homologue has at least 80% sequence identity to SEQ ID No. 26.~~

16. ~~A vaccine according to claim 7, wherein the polypeptide has the amino acid sequence of SEQ ID No. 29, SEQ ID No. 30, SEQ ID No. 31, or SEQ ID No. 32.~~

17. ~~A vaccine comprising a vector and an acceptable pharmaceutical or veterinary vehicle, the vector comprising a nucleotide sequence of the genome of porcine Porcine circovirus other than PWD circovirus and in that it is one of the primers of the following pair of primers:~~

a) ~~5' GTG TGC TCG ACA TTG GTC TG 3', and~~

~~5' TCG AAT GTT AAC TAC CTC AA 3'.~~

15. ~~Nucleotide sequence according to Claim 12, characterized in that said sequence is a specific consensus sequence of porcine circovirus other than PWD circovirus of type B and in that it is one of the primers of the following pair of primers:~~

a) ~~5' GGC GGC GCC ATC TGT AAC GGT TT 3' and~~

~~5' GAT GGC GCC GAA AGA CCG GTA TC 3'.~~

16. ~~Nucleotide sequence according to one of Claims 12 to 15, characterized in that it is labeled by a radioactive compound or by a nonradioactive compound.~~

17. ~~Nucleotide sequence, or a homologue or fragment thereof.~~

~~18. A vaccine according to one of Claims 12 to 16, characterized in that it is covalently or noneovalently immobilized on a support.~~

5 ~~18. Nucleotide sequence~~claim 17, further comprising a gene coding for an expression product capable of inhibiting or retarding the establishment or development of a genetic or acquired disease.

10 ~~19. A vaccine comprising a cell and an acceptable pharmaceutical or veterinary vehicle, wherein the cell is transformed with a nucleotide sequence of the genome of Porcine circovirus type B, or a homologue or fragment thereof.~~

~~20. A vaccine according to one of Claims 12 to 17, for the detection and/or the amplification of nucleic sequences.~~

15 ~~19. Cloning and/or expression vector, characterized in that it contains a nucleotide sequence according to one of Claims 1 to 5 and 11.~~

20 ~~20. Vector characterized in that it comprises a nucleotide sequence according to one of Claims 1 to 5 and 11, and in that it additionally comprises a gene of interest.~~

~~21. Viral pseudoparticle or particle generated from a vector according to one of Claims 19 and 20.~~

25 ~~22. Host cell, characterized in that it is transformed by a vector according to one of Claims 19 and 20, or a viral particle according to Claim 21.~~

~~23. Animal, comprising a cell transformed according to Claim 22.~~

30 ~~24. Procedure for preparation of a recombinant polypeptide, characterized in that it employs a vector according to one of Claims 19 and 20, a cell transformed by said vector and/or an animal comprising said transformed cell.~~



~~25. Procedure for preparation of a synthetic polypeptide, characterized in that it uses an amino acid sequence of claim 1, further comprising an adjuvant.~~

5        ~~21. A vaccine comprising a pharmaceutically acceptable vehicle and a single polypeptide, wherein the single polypeptide according to one of Claims 6 to 10.~~

~~26. Recombinant or synthetic polypeptide obtained by a procedure according to Claim 24 or 25.~~

10       ~~27. Hybrid polypeptide, characterized in that it contains at least the sequence of a polypeptide according to one of Claims 6 to 10 and 26, and a sequence of a polypeptide capable of inducing an immune response in man or animals.~~

15       ~~28. Hybrid polypeptide according to Claim 27, characterized in that it contains at least the sequence of a polypeptide according to one of Claims 6 to 10 and 26, and a sequence of a polypeptide capable of inducing a humoral and/or cellular response in man or animals.~~

20       ~~29. Nucleotide sequence coding for a hybrid polypeptide according to one of Claims 27 and 28.~~

~~30. Vector characterized in that it contains a nucleotide sequence according to Claim 29.~~

25       ~~31. Hybrid polypeptide according to one of Claims 27 and 28, characterized in that it is a recombinant polypeptide obtained by the employment of a vector according to Claim 30.~~

30       ~~32. Procedure for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, in a biological sample, characterized in that it comprises the following steps:~~

a) ~~contacting of the biological sample with a polypeptide according to one of Claims 6 to 10 and 26;~~

5

b) ~~demonstration of the antigen-antibody complex possibly formed.~~

33. ~~Procedure according to Claim 32 for the detection and/or identification of PWD circovirus of type B in a biological sample, characterized in that it comprises the following steps:~~

10

a) ~~contacting of the biological sample with a polypeptide according to Claim 10;~~

b) ~~demonstration of the antigen-antibody complex possibly formed.~~

15

34. ~~Kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:~~

20

a) ~~a polypeptide according to one of Claims 6 to 10 and 26;~~

b) ~~if need be, the reagents for the formation of the medium favorable to the immunological reaction;~~

c) ~~if need be, the reagents allowing demonstration of the antigen-antibody complexes possibly formed between the polypeptide(s) of the invention and the antibodies;~~

25

d) ~~if need be, a biological reference sample (negative control) devoid of antibodies recognized by said polypeptide;~~

30

e) ~~if need be, a biological reference sample (positive control) containing a predetermined quantity of antibodies recognized by said polypeptide.~~

35. ~~Mono or polyclonal antibodies, their fragments, or chimeric antibodies, characterized in that they are~~

~~capable of specifically recognizing a polypeptide according to one of Claims 6 to 10 and 26.~~

~~36. Antibody according to Claim 35, characterized in that it is a labeled antibody.~~

5 ~~37. Procedure for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, in a biological sample, characterized in that it comprises the following steps:~~

- 10 ~~a) contacting of the biological sample with an antibody according to one of Claims 35 or 36;~~  
~~b) demonstration of the antigen-antibody complex formed.~~

15 ~~38. Kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:~~

- 20 ~~a) a polyclonal or monoclonal antibody according to one of Claims 35 or 36;~~  
~~b) if need be, the reagents for the formation of the medium favorable to the immunological reaction;~~  
~~c) the reagents allowing the demonstration of the~~  
25 ~~antigen-antibody complexes produced by the immunological reaction.~~

~~39. Procedure for detection and/or identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD~~  
30 ~~circovirus of type B, in a biological sample, characterized in that it employs a nucleotide sequence according to one of Claims 12 to 18.~~

~~40. Procedure according to Claim 39, characterized in that it contains the following steps:~~

~~a) if need be, isolation of the DNA from the biological sample to be analyzed;~~

~~b) specific amplification of the DNA of PWD circovirus with the aid of at least one primer according to one of Claims 12 to 18;~~

~~c) demonstration of the amplification products.~~

~~41. Procedure according to Claim 39, characterized in that it comprises the following steps:~~

~~a) contacting of a nucleotide probe according to one of Claims 12 to 18 with a biological sample, the DNA contained in the biological sample having, if need be, previously been made accessible to hybridization under conditions allowing the hybridization of the probe with the DNA of the sample;~~

~~b) demonstration of the hybrid possibly formed between the nucleotide probe and the DNA of the biological sample.~~

~~42. Procedure according to Claim 39, characterized in that it comprises the following steps:~~

~~a) contacting of a nucleotide probe immobilized on a support according to Claim 17 with a biological sample, the DNA of the sample having, if need be, previously been made accessible to hybridization under conditions allowing the hybridization of the probe with the DNA of the sample;~~

~~b) contacting of the hybrid formed between the nucleotide probe immobilized on a support and the DNA contained in the biological sample, if need be after elimination of the DNA of the biological sample which has not hybridized with the probe, with a nucleotide probe labeled according to Claim 16;~~

~~c) demonstration of the novel hybrid formed in step b).~~

~~43. Procedure according to Claim 41 or 42, characterized in that, previously to step a), the DNA of~~

~~the biological sample is amplified with the aid of at least one primer according to one of Claims 12 to 15.~~

44. ~~Kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:~~

a) ~~a nucleotide probe according to one of Claims 12 to 18;~~

b) ~~if need be, the reagents necessary for the carrying out of a hybridization reaction;~~

c) ~~if need be, at least one primer according to one of Claims 12 to 18, as well as the reagents necessary for an amplification reaction of the DNA.~~

45. ~~Kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:~~

a) ~~a nucleotide probe, a so-called capture probe, according to Claim 17;~~

b) ~~an oligonucleotide probe, called a revealing probe, according to Claim 16;~~

c) ~~if need be, at least one primer according to one of Claims 12 to 18, as well as the reagents necessary for an amplification reaction of the DNA.~~

46. ~~Kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:~~

a) ~~at least one primer according to one of Claims 12 to 18;~~

- b) ~~if need be, the reagents necessary for carrying out a DNA amplification reaction;~~
- e) ~~if need be, a component allowing the sequence of the amplified fragment to be verified, more particularly an oligonucleotide probe according to one of Claims 12 to 18.~~ consists of SEQ ID No. 26.

5

22. A method of immunizing a mammal against piglet weight loss disease comprising administering to a mammal an effective amount of the vaccine of any one of claims 1-21.

10

Abstract of the Invention

~~47. Procedure or kit or set according to one of Claims 32 to 34, or 37 to 46, for the diagnosis of an infection by a PWD circovirus, by a porcine~~The genome sequences and the nucleotide sequences coding for the PWD circovirus other than a PWD polypeptides, such as the circovirus or by a porcine circovirus other than the PWD circovirus of type B.48. Use of a nucleotide sequence according to one of Claims 1 to 5 and 11, of a polypeptide according to one of Claims 6 to 10~~structural and 26, of an antibody according to one of Claims 35~~non-structural polypeptides, vectors including the sequences, and 36, of a cell according to Claim 22, cells and/or of an animal~~animals transformed according to Claim 23, by the vectors are provided. Methods for the selection of organic or inorganic~~detecting the nucleic acids or polypeptides, and kits for diagnosing infection by a PWD circovirus, also are provided. Method for selecting compounds capable of modulating, inducing or inhibiting the expression of genes, and/or of modifying the cellular replication of PWD circovirus or capable of inducing or of inhibiting in pigs the pathologies linked to an~~the viral infection by a PWD circovirus.~~are further provided. Pharmaceutical, including vaccines, compositions for preventing and/or treating viral infections caused by PWD circovirus and the use of vectors for preventing and/or treating diseases also are provided.

~~49. Compound selection method capable of binding to a polypeptide according to one of Claims 6 to 10 and 26, capable of binding to a nucleotide sequence according to one of Claims 1 to 5 and 11, or capable of recognizing an antibody according to Claim 35, and/or capable of~~



~~modulating, inducing or inhibiting the expression of genes, and/or of modifying the cellular replication of PWD circovirus, or capable of inducing or inhibiting in pigs the pathologies linked to an infection by a PWD circovirus, characterized in that it comprises the following steps:~~

5      ~~a) contacting of said compound with said polypeptide, said nucleotide sequence, or with a cell transformed according to Claim 22, and/or administration of said compound to an animal transformed according to Claim 23;~~

~~b) determination of the activity of said compound.~~

~~50. Compound capable of being selected by a method according to Claim 49.~~

10      ~~51. Pharmaceutical composition comprising a compound selected from the following compounds:~~

~~a) a nucleotide sequence according to one of Claims 1 to 5, 11 and 29;~~

~~b) a polypeptide according to one of Claims 6 to 10, 26 to 28 and 31;~~

15      ~~c) a vector or a viral particle according to one of Claims 19 to 21 and 30, or a cell according to Claim 22;~~

~~d) an antibody according to Claim 35; and~~

~~e) a compound according to Claim 50.~~

20      ~~52. Compound according to Claim 51, in combination with a pharmaceutically acceptable vehicle and, if need be, one or more adjuvants of the appropriate immunity.~~

~~53. Vaccine composition, characterized in that it comprises a compound selected from the following compounds:~~

25      ~~a) a nucleotide sequence according to one of Claims 1 to 5, 11 and 29;~~

b) ~~a polypeptide according to one of Claims 6 to 10, 26 to 28 and 31;~~

e) ~~a vector or a viral particle according to one of Claims 19 to 21 and 30; and~~

5 d) ~~a cell according to Claim 22.~~

54. ~~Vaccine composition according to Claim 53, characterized in that it comprises a mixture of at least two of said compounds and in that one of the two said compounds is related to the PWD circovirus of type A and the other is related to the PWD circovirus of type B.~~

10 55. ~~Vaccine composition, characterized in that it comprises at least one of the following compounds:~~

~~— a nucleotide sequence SEQ ID No. 11, SEQ ID No. 12, or one of their fragments;~~

15 ~~— a polypeptide of sequence SEQ ID No. 14, SEQ ID No. 15, or one of their fragments;~~

~~— a vector or a viral particle comprising a nucleotide sequence SEQ ID No. 11, SEQ ID No. 12, or one of their fragments;~~

20 ~~— a transformed cell capable of expressing a polypeptide of sequence SEQ ID No. 14, SEQ ID No. 15, or one of their fragments; or~~

~~— a mixture of at least two of said compounds.~~

25 56. ~~Vaccine composition according to Claim 54 or 55, characterized in that it comprises said mixture of at least two of said compounds as a combination product for simultaneous, separate or protracted use for the prevention or the treatment of infection by a PWD circovirus.~~

30 57. ~~Vaccine composition according to Claim 55 or 56, characterized in that said mixture comprises the following compounds:~~

~~— a pcDNA3 plasmid containing a nucleic acid of sequence SEQ ID No. 11;~~

~~\_\_\_\_\_ a pcDNA3 plasmid containing a nucleic acid of sequence SEQ ID No. 12;~~

~~\_\_\_\_\_ a pcDNA3 plasmid containing a nucleic acid coding for the GM-CSF protein;~~

5 ~~\_\_\_\_\_ a recombinant baculovirus containing a nucleic acid of sequence SEQ ID No. 11;~~

~~\_\_\_\_\_ a recombinant baculovirus containing a nucleic acid of sequence SEQ ID No. 12; and~~

10 ~~\_\_\_\_\_ if need be, an adjuvant of the appropriate immunity, especially the adjuvant AIF<sup>TM</sup>.~~

~~58. \_\_\_\_\_ Pharmaceutical composition according to one of Claims 51 to 57, for the prevention or the treatment of an infection by a PWD circovirus.~~

15 ~~59. \_\_\_\_\_ Pharmaceutical composition according to one of Claims 54 to 58 for the prevention or the treatment of an infection by the PWD circovirus of type B.~~

~~60. \_\_\_\_\_ Use of a composition according to one of Claims 51 to 59 for the preparation of a medicament intended for the prevention or the treatment of infection by a PWD circovirus.~~

20 ~~61. \_\_\_\_\_ Use of a composition according to one of Claims 54 to 57 for the preparation of a medicament intended for the prevention or the treatment of infection by the PWD circovirus of type B.~~

25 ~~62. \_\_\_\_\_ Vector according to one of Claims 19, 20 and 30, viral particle according to Claim 21, or cell according to Claim 22, for the treatment and/or the prevention of a disease by gene therapy.~~

30 ~~63. \_\_\_\_\_ Use of a vector according to Claims 19, 20 and 30, of a viral particle according to Claim 21, or of a cell according to Claim 22, for the preparation of a medicament intended for the treatment and/or the prevention of a disease by gene therapy.~~

Table 5: ~~Summary of the measurements carried out during experimental reproduction of PWD. (The values of the control animals are reported in brackets, the underlined values indicate a difference between infected animals and control animals)~~

5

Test	2	3	4	5	6	7
Measurement						
Status of the pigs	SPF	SPF	SPF	SPF	Conventional	Conventional
Age	CNEVA 9 weeks	field 6 weeks	CNEVA 5 weeks	CNEVA 5 weeks	5 weeks	6-7 weeks
Number	4	6	12	8	8	8
Inoculation route	Intratracheal route	Intratracheal route	Intratracheal + intramuscular route	Intratracheal + intramuscular route	Intratracheal + intramuscular route	Intratracheal + intramuscular route
Inoculum titer per pig	ND*	ND*	$10^{4.53}$ TCID <sub>50</sub> per ml: 1 ml IM + 5 ml IT	$10^{4.53}$ TCID <sub>50</sub> per ml: 1 ml IM + 5 ml IT	$10^{4.53}$ TCID <sub>50</sub> per ml: 1 ml IM + 5 ml IT	$10^{4.53}$ TCID <sub>50</sub> per ml: 1 ml IM + 5 ml IT
Start of hyperthermia	10 days post-infection	9-13 days post-infection	12-13 days post-infection	9-14 days post-infection	8-12 days post-infection	12 days post-infection
% of pigs in hyperthermia**	100%	83%	92%	100%	75%	88%
Number of days of hyperthermia per pig**	7	4-5	3-3	5-8	7-5	11-6
Maximum temperatures ***	40.4 to 41.7°C	40.6 to 42.3°C	40.2 to 41.6°C	40.3 to 40.8°C	40.6 to 42°C	40.2 to 41.9°C
Hyperthermia**** % per week						

Test	2	3	4	5	6	7
Measurement						
W1	<del>3.5 (3.5)</del>	<del>17 (36)</del>	<del>7 (5)</del>	<del>37 (17)</del>	<del>16 (17)</del>	<del>20 (28)</del>
W2	<del>42 (3.5)</del>	<del>7 (13)</del>	<del>13 (1)</del>	<del>21 (3)</del>	<del>52 (10)</del>	<del>37 (28)</del>
W3	<del>35 (3.5)</del>	<del>33 (10)</del>	<del>28 (7)</del>	<del>62 (2)</del>	<del>34 (12)</del>	<del>79 (17)</del>
W4	<del>21 (3.5)</del>	<del>28 (7)</del>	<del>5 (0)</del>	<del>6 (3)</del>	<del>25 (22)</del>	<del>55 (3)</del>
DMG:						
W1	<del>928 (1053)</del>	<del>417 (357)</del>	<del>564 (620)</del>	<del>650 (589)</del>	<del>401 (407)</del>	<del>509 (512)</del>
W2	<del>678 (1028)</del>	<del>428 (617)</del>	<del>503 (718)</del>	<del>612 (584)</del>	<del>294 (514)</del>	<del>410 (310)</del>
W3	<del>661 (1000)</del>	<del>771 (642)</del>	<del>381 (657)</del>	<del>520 (851)</del>	<del>375 (586)</del>	<del>435 (440)</del>
W4	<del>786 (1100)</del>	<del>550 (657)</del>	<del>764 (778)</del>	<del>641 (696)</del>	<del>473 (610)</del>	<del>451 (681)</del>
Contact pigs	<del>Yes to 100%</del>	<del>Yes to 75%</del>	<del>Not tested</del>	<del>Not tested</del>	<del>Not tested</del>	<del>Not tested</del>
transmission						
% of pulmonary lesions	25	75	0	25	25	12
% of ganglionic lesions	17	33	67	25	50	12

\* ND: not determined,

\*\* hyperthermia when the temperature is greater than 40°C,

\*\*\* range of maximum temperatures recorded at the individual level,

5 \*\*\*\* the percentage corresponds to the number of temperature recordings greater than 40°C divided by the total number of temperature recordings in the week on all of the pigs.

Table 10: Results of the evaluation as a diagnostic antigen of synthetic peptides encoded by the nucleic sequences ORF2 and ORF'2 of PWD circovirus of type A and B.



Peptide		Type	Position	AA sequence	Infected pig serum reactivity			Epitope specificity
					SPF	Conventional-1	Conventional-2	
		PWD			D0/D54	D0/D42	D0/D42	
Circovirus								
121		B	71-85	VDMRFENINDFLPPG	+/+,+++	+/+,+++	+/+,+++	Circovirus-B
177		B	70-84	NVNEELRFNIGQFLPP	+/+,++	+/+,++	+/+,++	Circovirus-B
131		B	115-129	QCDRGVGSAAVILDD	+/+,++	+/+,++	+/+,++	Circovirus-B
188		A	114-127	TSNQRGVGSTVIL	+/+,++	+/+,++	+/+,++	Circovirus-B
133		B	119-134	GVGSAAVILDDNVFTK	+/+,++	+/+,++	+/+,++	Circovirus-B
189		A	118-132	RCVGSTVILDAFV	+/+,++	+/+,++	+/+,++	Circovirus-B
146		B	171-185	FTIDYFQPNKKRNQL	+/+,++	+/+,++	+/+,++	Circovirus-A&B
202		A	170-184	DQTDWFQPNKKRNQ	+/+,+++	+/+,+++	+/+,+++	Circovirus-B
152		B	195-209	VDHVGGLCTAFENSIY	+/+,++	+/+,++	+/+,++	Circovirus-B
208		A	194-208	NVEHTGLCYALQNAI	+/+,++	+/+,++	+/+,++	Circovirus-B

+/+, ++, +++. Increasing intensities of the reactivities observed in Spot peptides on a nitrocellulose membrane. The porcine sera tested are from animals experimentally infected with the circovirus of type B within the animal houses of the CNEVA. Samples are taken from the animals before inoculation on d0 and 42 days or 54 days after inoculation, on d42, d54.



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CIRCOVIRUS SEQUENCES ASSOCIATED WITH PIGLET WEIGHT LOSS  
DISEASE (PWD)

5 The invention relates to the genomic sequence  
and nucleotide sequences coding for polypeptides of PWD  
circovirus, such as the structural and nonstructural  
polypeptides of said circovirus, as well as vectors  
including said sequences and cells or animals  
transformed by these vectors. The invention likewise  
10 relates to methods for detecting these nucleic acids or  
polypeptides and kits for diagnosing infection by the  
PWD circovirus. The invention is also directed at a  
method for selecting compounds capable of modulating  
the viral infection. The invention finally comprises  
15 pharmaceutical compositions, especially vaccines, for  
the prevention and/or the treatment of viral infections  
by PWD circovirus as well as the use of a vector  
according to the invention for the prevention and/or  
the treatment of diseases by gene therapy.

20 Piglet weight loss disease (PWD) or  
alternatively called fatal piglet wasting (FPW) has  
been widely described in North America (Harding, J.C.,  
1997), and authors have reported the existence of a  
relationship between this pathology and the presence of  
25 porcine circovirus (Daft, B. et al., 1996; Clark, E.G.,  
1997; Harding, J.C., 1997; Harding, J.C. and Clark,  
E.G., 1997; Nayar, G.P. et al., 1997). A porcine  
circovirus has already been demonstrated in established  
lines of cell cultures derived from pigs and  
30 chronically infected (Tischer, I., 1986, 1988, 1995;  
Dulac, G.C., 1989; Edwards, S., 1994; Allan, G.M., 1995  
and McNeilly, F., 1996). This virus, during  
experimental infection of piglets, does not prove  
pathogenic for pigs (Tischer, I., 1986, Horner, G.W.,  
35 1991) and its nucleotide sequence has been determined  
and characterized (Tischer, I., 1982; Meehan, B.M. et  
al., 1997; Mankertz, A., 1997). The porcine  
circovirus, called PCV virus, is part of the circovirus

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EXHIBIT B

genus of the circoviridae family (Murphy, F.A. et al., 1995) whose virion has a circular DNA of size between 1.7 and 2.3 kb, which DNA comprises three open reading frames (ORF1 to ORF3), coding for a replication protein  
5 REP involved in the initiation and termination phase of rolling circular replication (RCR) (Heyraud-Nitschke, F., et al., 1995; Harding, M.R. et al., 1993; Hanson, S.F. et al., 1995; Fontes, E.P.B. et al., 1994), coding for a capsid protein (Boulton, L.H. et al., 1997;  
10 Hackland, A.F. et al., 1994; Chu, P.W.G. et al., 1993 and coding for a nonstructural protein called a dissemination protein (Lazarowitz., S.G. et al., 1989).

The authors of the present invention have noticed that the clinical signs perceptible in pigs and  
15 linked to infection by the PWD circovirus are very distinctive. These manifestations in general appear in pigs of 8 to 12 weeks of age, weaned for 4 to 8 weeks. The first signs are hypotonia without it being possible to speak of prostration. Rapidly (48 hours), the flanks  
20 hollow, the line of the spine becomes apparent, and the pigs "blanch". These signs are in general accompanied by hyperthermia, anorexia and most often by respiratory signs (coughing, dyspnea, polypnea). Transitory diarrhea can likewise appear. The disease state phase  
25 lasts approximately one month at the end of which the rate of mortality varies from 5 to 20%. To these mortalities, it is expedient to add a variable proportion (5-10%) of cadaveric animals which are no longer able to present an economic future. It is to be  
30 noted that outside of this critical stage of the end of post-weaning, no anomaly appears on the farms. In particular, the reproductive function is totally maintained.

On the epidemiological level, the first signs  
35 of this pathology appeared at the start of 1995 in the east of the Côtes d'Armor department in France, and the farms affected are especially confined to this area of the department. In December 1996, the number of farms

concerned could not be evaluated with precision because of the absence of a specific laboratory diagnostic method or of an epidemiological surveillance system of the livestock. Based on the clinical facts as well as on results of postmortem examinations supplied by veterinarians, it is possible to estimate this number as several dozen (80-100). The contagiousness of the disease is weak to moderate. Cases are being reported outside the initial area and for the majority are following the transfer of animals coming from farms familiar with the problem. On the other hand, a characteristic of the condition is its strong remanence. Thus, farms which have been affected for a year are still affected in spite of the massive administration of therapeutics. Farms with clinical expression are drawn from various categories of specialization (breeders/fatteners, post-weaners/fatteners) and different economic structures are concerned. In addition, the disorders appear even in farms where the rules of animal husbandry are respected.

Numerous postmortem examinations have been carried out either on farms or in the laboratory. The elements of the lesional table are disparate. The most constant macroscopic lesions are pneumonia which sometimes appears in patchy form as well as hypertrophy of the lymphatic ganglia. The other lesions above all affect the thoracic viscera including, especially, pericarditis and pleurisy. However, arthritis and gastric ulcers are also observed. The lesions revealed in the histological examination are essentially situated at the pulmonary level (interstitial pneumonia), ganglionic level (lymphoid depletion of the lymph nodes, giant cells) and renal level (glomerulonephritis, vasculitis). The infectious agents have been the subject of wide research. It has been possible to exclude the intervention of pestiviruses and Aujeszky's disease. The disorders appear in the

seropositive PDRS (Porcine Dysgenic and Respiratory Syndrome, an infection linked to an arteriovirus) herds, but it has not been possible to establish the role of the latter in the genesis of the disorders (the majority of the farms in Brittany are PDRS seropositive).

The authors of the present invention, with the aim of identifying the etiological agent responsible for PWD, have carried out "contact" tests between piglets which are obviously "ill" and SPF pigs (specific pathogen-free) from CNEVA (Centre National d'Etudes Vétérinaires et Alimentaires, France). These tests allow the development of signs comparable to those observed on the farm to be observed in protected animal houses. The discrete signs such as moderate hyperthermia, anorexia and intermittent diarrhea appeared after one week of contact. It must be noted that the PDRS virus only diffused subsequent to the clinical signs. In addition, inoculations of organ homogenates of sick animals to healthy pigs allowed signs related to those observed on the farms to be reproduced, although with a lower incidence, linked to the favorable conditions of upkeep of the animals in the experimental installations.

Thus, the authors of the present invention have been able to demonstrate that the pathological signs appear as a well-defined entity affecting the pig at a particular stage of its growth.

This pathology has never been described in France. However, sparse information, especially Canadian, relates to similar facts.

The disorders cannot be mastered with the existing therapeutics.

The data collected both on the farm and by experimentation have allowed the following points to be highlighted:

- PWD is transmissible but its contagiousness is not very high,

- its etiological origin is of infectious and probably viral nature,

- PWD has a persistent character in the affected farms.

Considerable economic consequences ensue for  
5 the farms.

Thus, there is currently a significant need for a specific and sensitive diagnostic, whose production is practical and rapid, allowing the early detection of the infection.

10 A reliable, sensitive and practical test which allows the distinction between strains of porcine circovirus (PCV) is thus strongly desirable.

On the other hand, a need for efficient and well-tolerated treatment of infections with PWD  
15 circovirus likewise remains desirable, no vaccine currently being available against PWD circovirus.

Concerning PWD circovirus, it will probably be necessary to understand the role of the immune defense in the physiology and the pathology of the disease to  
20 develop satisfactory vaccines.

Fuller information concerning the biology of these strains, their interactions with their hosts, the associated infectivity phenomena and those of escape from the immune defenses of the host especially, and  
25 finally their implication in the development of associated pathologies, will allow a better understanding of these mechanisms. Taking into account the facts which have been mentioned above and which show in particular the limitations of combatting  
30 infection by the PWD circovirus, it is thus essential today on the one hand to develop molecular tools, especially starting from a better genetic knowledge of the PWD circovirus, but likewise to perfect novel preventive and therapeutic treatments, novel methods of  
35 diagnosis and specific, efficacious and tolerated novel vaccine strategies. This is precisely the subject of the present invention.

The present invention relates to nucleotide sequences of the genome of PWD circovirus selected from the sequences SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 9, SEQ ID No. 10 or one of their fragments.

5           The nucleotide sequences of sequences SEQ ID No. 1 and SEQ ID No. 2 correspond respectively to the genome sequence of the strand of (+) polarity and of the strand of (-) polarity of the PWD circovirus of type A (or PCVA), the sequence SEQ ID No. 2 being  
10 represented according to the orientation 5'→3'.

          The nucleotide sequences of sequences SEQ ID No. 9 and SEQ ID No. 10 correspond respectively to the genome sequence of the strand of (+) polarity and of the strand of (-) polarity of the PWD circovirus of  
15 type B (or PCVB), the sequence SEQ ID No. 10 being represented according to the orientation 5'→3'.

          The present invention likewise relates to nucleotide sequences, characterized in that they are selected from:

- 20   a)   a nucleotide sequence of a specific fragment of the sequence SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 9, SEQ ID No. 10 or one of their fragments;
- b)   a nucleotide sequence homologous to a nucleotide sequence such as defined in a);
- 25   c)   a nucleotide sequence complementary to a nucleotide sequence such as defined in a) or b), and a nucleotide sequence of their corresponding RNA;
- d)   a nucleotide sequence capable of hybridizing under  
30 stringent conditions with a sequence such as defined in a), b) or c);
- e)   a nucleotide sequence comprising a sequence such as defined in a), b), c) or d); and
- f)   a nucleotide sequence modified by a nucleotide  
35 sequence such as defined in a), b), c), d) or e).

          Nucleotide, polynucleotide or nucleic acid sequence will be understood according to the present invention as meaning both a double-stranded or single-



stranded DNA in the monomeric and dimeric (so-called in tandem) forms and the transcription products of said DNAs.

It must be understood that the present invention does not relate to the genomic nucleotide sequences taken in their natural environment, that is to say in the natural state. It concerns sequences which it has been possible to isolate, purify or partially purify, starting from separation methods such as, for example, ion-exchange chromatography, by exclusion based on molecular size, or by affinity, or alternatively fractionation techniques based on solubility in different solvents, or starting from methods of genetic engineering such as amplification, cloning and subcloning, it being possible for the sequences of the invention to be carried by vectors.

The nucleotide sequences SEQ ID No. 1 and SEQ ID No. 9 were obtained by sequencing of the genome by the Sanger method.

Nucleotide sequence fragment according to the invention will be understood as designating any nucleotide fragment of the PWD circovirus, type A or B, of length of at least 8 nucleotides, preferably at least 12 nucleotides, and even more preferentially at least 20 consecutive nucleotides of the sequence from which it originates.

Specific fragment of a nucleotide sequence according to the invention will be understood as designating any nucleotide fragment of the PWD circovirus, type A or B, having, after alignment and comparison with the corresponding fragments of known porcine circoviruses, at least one nucleotide or base of different nature. For example, the specific nucleotide fragments of the PWD circovirus of type A can easily be determined by referring to Figure 3 of the present invention in which the nucleotides or bases of the sequence SEQ ID No. 1 (circopordfp) are shown which are of different nature, after alignment of said

sequence SEQ ID No. 1 with the other two sequences of known porcine circovirus (circopormeeh and circopormank).

Homologous nucleotide sequence in the sense of the present invention is understood as meaning a nucleotide sequence having at least a percentage identity with the bases of a nucleotide sequence according to the invention of at least 80%, preferably 90% or 95%, this percentage being purely statistical and it being possible to distribute the differences between the two nucleotide sequences at random and over the whole of their length.

Specific homologous nucleotide sequence in the sense of the present invention is understood as meaning a homologous nucleotide sequence having at least one nucleotide sequence of a specific fragment, such as defined above. Said "specific" homologous sequences can comprise, for example, the sequences corresponding to the genomic sequence or to the sequences of its fragments representative of variants of PWD circovirus of type A or B. These specific homologous sequences can thus correspond to variations linked to mutations within strains of PWD circovirus of type A and B, and especially correspond to truncations, substitutions, deletions and/or additions of at least one nucleotide. Said homologous sequences can likewise correspond to variations linked to the degeneracy of the genetic code.

In the present description, PWD circovirus will be understood as designating the circoviruses associated with piglet weight loss disease (PWD) of type A (PCVA) or type B (PCVB), defined below by their genomic sequence, as well as the circoviruses whose nucleic sequences are homologous to the sequences of PWD circoviruses of type A or B, such as in particular the circoviruses corresponding to variants of the type A or of the type B.

Complementary nucleotide sequence of a sequence of the invention is understood as meaning any DNA whose nucleotides are complementary to those of the sequence of the invention, and whose orientation is reversed (antiparallel sequence).

Hybridization under conditions of stringency with a nucleotide sequence according to the invention is understood as meaning a hybridization under conditions of temperature and ionic strength chosen in such a way that they allow the maintenance of the hybridization between two fragments of complementary DNA.

By way of illustration, conditions of great stringency of the hybridization step with the aim of defining the nucleotide fragments described above are advantageously the following.

The hybridization is carried out at a preferential temperature of 65°C in the presence of SSC buffer, 1 × SSC corresponding to 0.15 M NaCl and 0.05 M Na citrate. The washing steps, for example, can be the following:

- 2 × SSC, at ambient temperature followed by two washes with 2 × SSC, 0.5% SDS at 65°C; 2 × 0.5 × SSC, 0.5% SDS; at 65°C for 10 minutes each.

The conditions of intermediate stringency, using, for example, a temperature of 42°C in the presence of a 2 × SSC buffer, or of less stringency, for example a temperature of 37°C in the presence of a 2 × SSC buffer, respectively require a globally less significant complementarity for the hybridization between the two sequences.

The stringent hybridization conditions described above for a polynucleotide with a size of approximately 350 bases will be adapted by the person skilled in the art for oligonucleotides of greater or smaller size, according to the teaching of Sambrook et al., 1989.

Among the nucleotide sequences according to the invention, those are likewise preferred which can be used as a primer or probe in methods allowing the homologous sequences according to the invention to be  
5 obtained, these methods, such as the polymerase chain reaction (PCR), nucleic acid cloning and sequencing, being well known to the person skilled in the art.

Among said nucleotide sequences according to the invention, those are again preferred which can be  
10 used as a primer or probe in methods allowing the presence of PWD circovirus or one of its variants such as defined below to be diagnosed.

The nucleotide sequences according to the invention capable of modulating, of inhibiting or of  
15 inducing the expression of PWD circovirus gene, and/or capable of modulating the replication cycle of PWD circovirus in the host cell and/or organism are likewise preferred. Replication cycle will be understood as designating the invasion and the  
20 multiplication of PWD circovirus, and its propagation from host cell to host cell in the host organism.

Among said nucleotide sequences according to the invention, those corresponding to open reading frames, called ORF sequences, and coding for  
25 polypeptides, such as, for example, the sequences SEQ ID No. 3 (ORF1), SEQ ID No. 4 (ORF2) and SEQ ID No. 5 (ORF3) respectively corresponding to the nucleotide sequences between the positions 47 and 985 determined with respect to the position of the nucleotides on the  
30 sequence SEQ ID No. 1, the positions 1723 and 1022 and the positions 658 and 38 with respect to the position of the nucleotides on the sequence SEQ ID No. 2 (represented according to the orientation 3'→5'), the ends being included, or alternatively the sequences SEQ  
35 ID No. 11 (ORF'1), SEQ ID No. 12 (ORF'2) and SEQ ID No. 13 (ORF'3), respectively corresponding to the sequences between the positions 51 and 995 determined with respect to the position of the nucleotides on the

sequence SEQ ID No. 9, the positions 1734 and 1033 and the positions 670 and 357, the positions being determined with respect to the position of the nucleotides on the sequence SEQ ID No. 10 (represented according to the orientation 3'→5'), the ends being included, are finally preferred.

The nucleotide sequence fragments according to the invention can be obtained, for example, by specific amplification, such as PCR, or after digestion with appropriate restriction enzymes of nucleotide sequences according to the invention, these methods in particular being described in the work of Sambrook et al., 1989. Said representative fragments can likewise be obtained by chemical synthesis when their size is not very large and according to methods well known to persons skilled in the art.

Modified nucleotide sequence will be understood as meaning any nucleotide sequence obtained by mutagenesis according to techniques well known to the person skilled in the art, and containing modifications with respect to the normal sequences according to the invention, for example mutations in the regulatory and/or promoter sequences of polypeptide expression, especially leading to a modification of the rate of expression of said polypeptide or to a modulation of the replicative cycle.

Modified nucleotide sequence will likewise be understood as meaning any nucleotide sequence coding for a modified polypeptide such as defined below.

The present invention relates to nucleotide sequences of PWD circovirus according to the invention, characterized in that they are selected from the sequences SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13 or one of their fragments.

The invention likewise relates to nucleotide sequences characterized in that they comprise a nucleotide sequence selected from:

a) a nucleotide sequence SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13 or one of their fragments;

5 b) a nucleotide sequence of a specific fragment of a sequence such as defined in a);

c) a homologous nucleotide sequence having at least 80% identity with a sequence such as defined in a) or b);

10 d) a complementary nucleotide sequence or sequence of RNA corresponding to a sequence such as defined in a), b) or c); and

e) a nucleotide sequence modified by a sequence such as defined in a), b), c) or d).

15 As far as homology with the nucleotide sequences SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13 or one of their fragments is concerned, the homologous, especially specific, sequences having a percentage identity with one of the sequences SEQ ID No. 3, SEQ ID No. 4, SEQ ID  
20 No. 5, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13 or one of their fragments of at least 80%, preferably 90% or 95%, are preferred. Said specific homologous sequences can comprise, for example, the sequences corresponding to the sequences ORF1, ORF2, ORF3, ORF'1,  
25 ORF'2 and ORF'3 of PWD circovirus variants of type A or of type B. In the same manner, these specific homologous sequences can correspond to variations linked to mutations within strains of PWD circovirus of type A or of type B and especially correspond to  
30 truncations, substitutions, deletions and/or additions of at least one nucleotide.

Among nucleotide sequences according to the invention, the sequence SEQ ID No. 11 which has a homology having more than 80% identity with the  
35 sequence SEQ ID No. 3, as well as the sequence SEQ ID No. 12, are especially preferred.

Preferably, the invention relates to the nucleotide sequences according to the invention,



characterized in that they comprise a nucleotide sequence selected from the following sequences:

- a) 170 5' TGTGGCGA 3';
- b) 450 5' AGTTTCCT 3';
- 5 c) 1026 5' TCATTTAGAGGGTCTTTCAG 3';
- d) 1074 5' GTCAACCT 3';
- e) 1101 5' GTGGTTGC 3';
- f) 1123 5' AGCCCAGG 3';
- g) 1192 5' TTGGCTGG 3';
- 10 h) 1218 5' TCTAGCTCTGGT 3';
- i) 1501 5' ATCTCAGCTCGT 3';
- j) 1536 5' TGTCCTCCTCTT 3';
- k) 1563 5' TCTCTAGA 3';
- l) 1623 5' TGTACCAA 3';
- 15 m) 1686 5' TCCGTCTT 3';

and their complementary sequences.

In the list of nucleotide sequences a)-m) above, the underlined nucleotides are mutated with respect to the two known sequences of circovirus which are nonpathogenic to pigs. The number preceding the nucleotide sequence represents the position of the first nucleotide of said sequence in the sequence SEQ ID No. 1.

The invention comprises the polypeptides encoded by a nucleotide sequence according to the invention, preferably a polypeptide whose sequence is represented by a fragment, especially a specific fragment, of one of the six sequences of amino acids represented in Figure 2, these six amino acid sequences corresponding to the polypeptides which can be encoded according to one of the three possible reading frames of the sequence SEQ ID No. 1 or of the sequence SEQ ID No. 2, or a polypeptide whose sequence is represented by a fragment, especially a specific fragment, of one of the six sequences of amino acids shown in Figure 8, these six sequences of amino acids corresponding to the polypeptides which can be encoded according to one of



the three possible reading frames of the sequence SEQ ID No. 9 or of the sequence SEQ ID No. 10.

The invention likewise relates to the polypeptides, characterized in that they comprise a polypeptide selected from the amino acid sequences SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16 or one of their fragments.

Among the polypeptides according to the invention, the polypeptide of amino acid sequence SEQ ID No. 14 which has a homology having more than 80% identity with the sequence SEQ ID No. 6, as well as the polypeptide of sequence SEQ ID No. 15, are especially preferred.

The invention also relates to the polypeptides, characterized in that they comprise a polypeptide selected from:

- a) a specific fragment of at least 5 amino acids of a polypeptide of an amino acid sequence according to the invention;
- b) a polypeptide homologous to a polypeptide such as defined in a);
- c) a specific biologically active fragment of a polypeptide such as defined in a) or b); and
- d) a polypeptide modified by a polypeptide such as defined in a), b) or c).

Among the polypeptides according to the invention, the polypeptides of amino acid sequences SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19 and SEQ ID No. 20 are also preferred, these polypeptides being especially capable of specifically recognizing the antibodies produced during infection by the PWD circovirus of type B. These polypeptides thus have epitopes specific for the PWD circovirus of type B and can thus be used in particular in the diagnostic field or as immunogenic agent to confer protection in pigs against infection by PWD circovirus, especially of type B.

In the present description, the terms polypeptide, peptide and protein are interchangeable.

It must be understood that the invention does not relate to the polypeptides in natural form, that is to say that they are not taken in their natural environment but that they can be isolated or obtained by purification from natural sources, or else obtained by genetic recombination, or alternatively by chemical synthesis and that they can thus contain unnatural amino acids, as will be described below.

Polypeptide fragment according to the invention is understood as designating a polypeptide containing at least 5 amino acids, preferably 10 amino acids or 15 amino acids.

In the present invention, specific polypeptide fragment is understood as designating the polypeptide fragment encoded by a specific fragment nucleotide sequence according to the invention.

Homologous polypeptide will be understood as designating the polypeptides having, with respect to the natural polypeptide, certain modifications such as, in particular, a deletion, addition or substitution of at least one amino acid, a truncation, a prolongation, a chimeric fusion, and/or a mutation. Among the homologous polypeptides, those are preferred whose amino acid sequence has at least 80%, preferably 90%, homology with the sequences of amino acids of polypeptides according to the invention.

Specific homologous polypeptide will be understood as designating the homologous polypeptides such as defined above and having a specific fragment of polypeptide according to the invention.

In the case of a substitution, one or more consecutive or nonconsecutive amino acids are replaced by "equivalent" amino acids. The expression "equivalent" amino acid is directed here at designating any amino acid capable of being substituted by one of the amino acids of the base structure without, however,

essentially modifying the biological activities of the corresponding peptides and such that they will be defined by the following.

These equivalent amino acids can be determined either by depending on their structural homology with the amino acids which they substitute, or on results of comparative tests of biological activity between the different polypeptides, which are capable of being carried out.

By way of example, the possibilities of substitutions capable of being carried out without resulting in an extensive modification of the biological activity of the corresponding modified polypeptides will be mentioned, the replacement, for example, of leucine by valine or isoleucine, of aspartic acid by glutamic acid, of glutamine by asparagine, of arginine by lysine etc., the reverse substitutions naturally being envisageable under the same conditions.

The specific homologous polypeptides likewise correspond to polypeptides encoded by the specific homologous nucleotide sequences such as defined above and thus comprise in the present definition the polypeptides which are mutated or correspond to variants which can exist in PWD circovirus, and which especially correspond to truncations, substitutions, deletions and/or additions of at least one amino acid residue.

Specific biologically active fragment of a polypeptide according to the invention will be understood in particular as designating a specific polypeptide fragment, such as defined above, having at least one of the characteristics of polypeptides according to the invention, especially in that it is:

- capable of inducing an immunogenic reaction directed against a PWD circovirus; and/or

- capable of being recognized by a specific antibody of a polypeptide according to the invention; and/or
- capable of linking to a polypeptide or to a nucleotide sequence of PWD circovirus; and/or
- capable of exerting a physiological activity, even partial, such as, for example, a dissemination or structural (capsid) activity; and/or
- capable of modulating, of inducing or of inhibiting the expression of PWD circovirus gene or one of its variants, and/or capable of modulating the replication cycle of PWD circovirus in the cell and/or the host organism.

The polypeptide fragments according to the invention can correspond to isolated or purified fragments naturally present in a PWD circovirus or correspond to fragments which can be obtained by cleavage of said polypeptide by a proteolytic enzyme, such as trypsin or chymotrypsin or collagenase, or by a chemical reagent, such as cyanogen bromide (CNBr) or alternatively by placing said polypeptide in a very acidic environment, for example at pH 2.5. Such polypeptide fragments can likewise just as easily be prepared by chemical synthesis, from hosts transformed by an expression vector according to the invention containing a nucleic acid allowing the expression of said fragments, placed under the control of appropriate regulation and/or expression elements.

"Modified polypeptide" of a polypeptide according to the invention is understood as designating a polypeptide obtained by genetic recombination or by chemical synthesis as will be described below, having at least one modification with respect to the normal sequence. These modifications will especially be able to bear on amino acids at the origin of a specificity, of pathogenicity and/or of virulence, or at the origin of the structural conformation, and of the capacity of membrane insertion of the polypeptide according to the

invention. It will thus be possible to create polypeptides of equivalent, increased or decreased activity, and of equivalent, narrower, or wider specificity. Among the modified polypeptides, it is  
5 necessary to mention the polypeptides in which up to 5 amino acids can be modified, truncated at the N- or C-terminal end, or even deleted or added.

As is indicated, the modifications of the polypeptide will especially have as objective:

- 10 - to render it capable of modulating, of inhibiting or of inducing the expression of PWD circovirus gene and/or capable of modulating the replication cycle of PWD circovirus in the cell and/or the host organism,
- 15 - of allowing its incorporation into vaccine compositions,
- of modifying its bioavailability as a compound for therapeutic use.

The methods allowing said modulations on  
20 eukaryotic or prokaryotic cells to be demonstrated are well known to the person skilled in the art. It is likewise well understood that it will be possible to use the nucleotide sequences coding for said modified polypeptides for said modulations, for example through  
25 vectors according to the invention and described below, in order, for example, to prevent or to treat the pathologies linked to the infection.

The preceding modified polypeptides can be obtained by using combinatorial chemistry, in which it  
30 is possible to systematically vary parts of the polypeptide before testing them on models, cell cultures or microorganisms for example, to select the compounds which are most active or have the properties sought.

35 Chemical synthesis likewise has the advantage of being able to use:

- unnatural amino acids, or
- nonpeptide bonds.

Thus, in order to improve the duration of life of the polypeptides according to the invention, it may be of interest to use unnatural amino acids, for example in D form, or else amino acid analogs, especially sulfur-containing forms, for example.

Finally, it will be possible to integrate the structure of the polypeptides according to the invention, its specific or modified homologous forms, into chemical structures of polypeptide type or others. Thus, it may be of interest to provide at the N- and C-terminal ends compounds not recognized by the proteases.

The nucleotide sequences coding for a polypeptide according to the invention are likewise part of the invention.

The invention likewise relates to nucleotide sequences utilizable as a primer or probe, characterized in that said sequences are selected from the nucleotide sequences according to the invention.

Among the pairs of nucleotide sequences utilizable as a pair of primers according to the invention, the pairs of primers selected from the following pairs are preferred:

- a) 5' GTG TGC TCG ACA TTG GTG TG 3', and  
5' TGG AAT GTT AAC GAG CTG AG 3';
- b) 5' GTG TGC TCG ACA TTG GTG TG 3', and  
5' CTC GCA GCC ATC TTG GAA TG 3';
- c) 5' CGC GCG TAA TAC GAC TCA CT 3', and  
5' GTG TGC TCG ACA TTG GTG TG 3';
- d) 5' CGC GCG TAA TAC GAC TCA CT 3', and  
5' CTC GCA GCC ATC TTG GAA TG 3'; and
- e) 5' CCT GTC TAC TGC TGT GAG TAC CTT GT 3', and  
5' GCA GTA GAC AGG TCA CTC CGT TGT CC 3'.

The cloning and the sequencing of the PWD circovirus, type A and B, has allowed it to be identified, after comparative analysis with the nucleotide sequences of other porcine circoviruses, that, among the sequences of fragments of these nucleic

acids, were those which are strictly specific to the PWD circovirus of type A, of type B or of type A and B, and those which correspond to a consensus sequence of porcine circoviruses other than the PWD circoviruses of type A and/or B.

There is likewise a great need for nucleotide sequences utilizable as a primer or probe specific to the whole of the other known and nonpathogenic porcine circoviruses.

Said consensus nucleotide sequences specific to all circoviruses, other than PWD circovirus of type A and B, are easily identifiable from Figure 3 and the sequence SEQ ID No. 9, and are part of the invention.

Among said consensus nucleotide sequences, that which is characterized in that it is part of the following pair of primers is preferred:

a) 5' GTG TGC TCG ACA TTG GTG TG 3', and  
5' TGG AAT GTT AAC TAC CTC AA 3'.

The invention likewise comprises a nucleotide sequence according to the invention, characterized in that said sequence is a specific consensus sequence of porcine circovirus other than PWD circovirus of type B and in that it is one of the primers of the following pairs of primers:

a) 5' GGC GGC GCC ATC TGT AAC GGT TT 3', and  
5' GAT GGC GCC GAA AGA CGG GTA TC 3'.

It is well understood that the present invention likewise relates to specific polypeptides of known porcine circoviruses other than PWD circovirus, encoded by said consensus nucleotide sequences, capable of being obtained by purification from natural polypeptides, by genetic recombination or by chemical synthesis by procedures well known to the person skilled in the art and such as described in particular below. In the same manner, the labeled or unlabeled mono- or polyclonal antibodies directed against said specific polypeptides encoded by said consensus nucleotide sequences are also part of the invention.



It will be possible to use said consensus nucleotide sequences, said corresponding polypeptides as well as said antibodies directed against said polypeptides in procedures or sets for detection and/or  
5 identification such as described below, in place of or in addition to nucleotide sequences, polypeptides or antibodies according to the invention, specific to PWD circovirus type A and/or B.

These protocols have been improved for the  
10 differential detection of the circular monomeric forms of specific replicative forms of the virion or of the DNA in replication and the dimeric forms found in so-called in-tandem molecular constructs.

The invention additionally relates to the use  
15 of a nucleotide sequence according to the invention as a primer or probe for the detection and/or the amplification of nucleic acid sequences.

The nucleotide sequences according to the invention can thus be used to amplify nucleotide  
20 sequences, especially by the PCR technique (polymerase chain reaction) (Erlich, 1989; Innis et al., 1990; Rolfs et al., 1991; and White et al., 1997).

These oligodeoxyribonucleotide or oligoribonucleotide primers advantageously have a length of at  
25 least 8 nucleotides, preferably of at least 12 nucleotides, and even more preferentially at least 20 nucleotides.

Other amplification techniques of the target nucleic acid can be advantageously employed as  
30 alternatives to PCR.

The nucleotide sequences of the invention, in particular the primers according to the invention, can likewise be employed in other procedures of amplification of a target nucleic acid, such as:

35 - the TAS technique (Transcription-based Amplification System), described by Kwoh et al. in 1989;

- the 3SR technique (Self-Sustained Sequence Replication), described by Guatelli et al. in 1990;
- the NASBA technique (Nucleic Acid Sequence Based Amplification), described by Kievitis et al. in 1991;
- the SDA technique (Strand Displacement Amplification) (Walker et al., 1992);
- the TMA technique (Transcription Mediated Amplification).

The polynucleotides of the invention can also be employed in techniques of amplification or of modification of the nucleic acid serving as a probe, such as:

- the LCR technique (Ligase Chain Reaction), described by Landegren et al. in 1988 and improved by Barany et al. in 1991, which employs a thermostable ligase;
- the RCR technique (Repair Chain Reaction), described by Segev in 1992;
- the CPR technique (Cycling Probe Reaction), described by Duck et al. in 1990;
- the amplification technique with Q-beta replicase, described by Miele et al. in 1983 and especially improved by Chu et al. in 1986, Lizardi et al. in 1988, then by Burg et al. as well as by Stone et al. in 1996.

In the case where the target polynucleotide to be detected is possibly an RNA, for example an mRNA, it will be possible to use, prior to the employment of an amplification reaction with the aid of at least one primer according to the invention or to the employment of a detection procedure with the aid of at least one probe of the invention, an enzyme of reverse transcriptase type in order to obtain a cDNA from the RNA contained in the biological sample. The cDNA obtained will thus serve as a target for the primer(s)

or the probe(s) employed in the amplification or detection procedure according to the invention.

The detection probe will be chosen in such a manner that it hybridizes with the target sequence or  
5 the amplicon generated from the target sequence. By way of sequence, such a probe will advantageously have a sequence of at least 12 nucleotides, in particular of at least 20 nucleotides, and preferably of at least 100 nucleotides.

10 The invention also comprises the nucleotide sequences utilizable as a probe or primer according to the invention, characterized in that they are labeled with a radioactive compound or with a nonradioactive compound.

15 The unlabeled nucleotide sequences can be used directly as probes or primers, although the sequences are generally labeled with a radioactive element ( $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^3\text{H}$ ,  $^{125}\text{I}$ ) or with a nonradioactive molecule (biotin, acetylaminofluorene, digoxigenin, 5-bromodeoxyuridine,  
20 fluorescein) to obtain probes which are utilizable for numerous applications.

Examples of nonradioactive labeling of nucleotide sequences are described, for example, in French Patent No. 78.10975 or by Urdea et al. or by  
25 Sanchez-Pescador et al. in 1988.

In the latter case, it will also be possible to use one of the labeling methods described in patents FR-2 422 956 and FR-2 518 755.

The hybridization technique can be carried out  
30 in various manners (Matthews et al., 1988). The most general method consists in immobilizing the nucleic acid extract of cells on a support (such as nitrocellulose, nylon, polystyrene) and in incubating, under well-defined conditions, the immobilized target  
35 nucleic acid with the probe. After hybridization, the excess of probe is eliminated and the hybrid molecules formed are detected by the appropriate method

(measurement of the radioactivity, of the fluorescence or of the enzymatic activity linked to the probe).

The invention likewise comprises the nucleotide sequences according to the invention, characterized in that they are immobilized on a support, covalently or noncovalently.

According to another advantageous mode of employing nucleotide sequences according to the invention, the latter can be used immobilized on a support and can thus serve to capture, by specific hybridization, the target nucleic acid obtained from the biological sample to be tested. If necessary, the solid support is separated from the sample and the hybridization complex formed between said capture probe and the target nucleic acid is then detected with the aid of a second probe, a so-called detection probe, labeled with an easily detectable element.

Another subject of the present invention is a vector for the cloning and/or expression of a sequence, characterized in that it contains a nucleotide sequence according to the invention.

The vectors according to the invention, characterized in that they contain the elements allowing the expression and/or the secretion of said nucleotide sequences in a determined host cell, are likewise part of the invention.

The vector must then contain a promoter, signals of initiation and termination of translation, as well as appropriate regions of regulation of transcription. It must be able to be maintained stably in the host cell and can optionally have particular signals specifying the secretion of the translated protein. These different elements are chosen as a function of the host cell used. To this end, the nucleotide sequences according to the invention can be inserted into autonomous replication vectors within the chosen host, or integrated vectors of the chosen host.

Such vectors will be prepared according to the methods currently used by the person skilled in the art, and it will be possible to introduce the clones resulting therefrom into an appropriate host by  
5 standard methods, such as, for example, lipofection, electroporation and thermal shock.

The vectors according to the invention are, for example, vectors of plasmid or viral origin.

A preferred vector for the expression of  
10 polypeptides of the invention is baculovirus.

The vector pBS KS in which is inserted the in-tandem DNA sequence of the PWD circovirus type A (or DFP) as deposited at the CNCM on 3 July 1997, under the number I-1891, is likewise preferred.

15 These vectors are useful for transforming host cells in order to clone or to express the nucleotide sequences of the invention.

The invention likewise comprises the host cells transformed by a vector according to the invention.

20 These cells can be obtained by the introduction into host cells of a nucleotide sequence inserted into a vector such as defined above, then the culturing of said cells under conditions allowing the replication and/or expression of the transfected nucleotide  
25 sequence.

The host cell can be selected from prokaryotic or eukaryotic systems, such as, for example, bacterial cells (Olins and Lee, 1993), but likewise yeast cells (Buckholz, 1993), as well as animal cells, in  
30 particular the cultures of mammalian cells (Edwards and Aruffo, 1993), and especially Chinese hamster ovary (CHO) cells, but likewise the cells of insects in which it is possible to use procedures employing baculoviruses, for example (Luckow, 1993).

35 A preferred host cell for the expression of the proteins of the invention is constituted by sf9 insect cells.

A more preferred host cell according to the invention is E. coli, such as deposited at the CNCM on 3 July 1997, under the number I-1891.

5 The invention likewise relates to animals comprising one of said transformed cells according to the invention.

10 The obtainment of transgenic animals according to the invention overexpressing one or more of the genes of PWD circovirus or part of the genes will be preferably carried out in rats, mice or rabbits according to methods well known to the person skilled in the art, such as by viral or nonviral transfections. It will be possible to obtain the transgenic animals overexpressing one or more of said genes by  
15 transfection of multiple copies of said genes under the control of a strong promoter of ubiquitous nature, or selective for one type of tissue. It will likewise be possible to obtain the transgenic animals by homologous recombination in embryonic cell strains, transfer of  
20 these cell strains to embryos, selection of the affected chimeras at the level of the reproductive lines, and growth of said chimeras.

The transformed cells as well as the transgenic animals according to the invention are utilizable in  
25 procedures for preparation of recombinant polypeptides.

It is today possible to produce recombinant polypeptides in relatively large quantity by genetic engineering using the cells transformed by expression vectors according to the invention or using transgenic  
30 animals according to the invention.

The procedures for preparation of a polypeptide of the invention in recombinant form, characterized in that they employ a vector and/or a cell transformed by a vector according to the invention and/or a transgenic  
35 animal comprising one of said transformed cells according to the invention, are themselves comprised in the present invention.

Among said procedures for preparation of a polypeptide of the invention in recombinant form, the preparation procedures employing a vector, and/or a cell transformed by said vector and/or a transgenic animal comprising one of said transformed cells, containing a nucleotide sequence according to the invention coding for a polypeptide of PWD circovirus, are preferred.

The recombinant polypeptides obtained as indicated above can just as well be present in glycosylated form as in nonglycosylated form and can or cannot have the natural tertiary structure.

A preferred variant consists in producing a recombinant polypeptide fused to a "carrier" protein (chimeric protein). The advantage of this system is that it allows a stabilization of and a decrease in the proteolysis of the recombinant product, an increase in the solubility in the course of renaturation in vitro and/or a simplification of the purification when the fusion partner has an affinity for a specific ligand.

More particularly, the invention relates to a procedure for preparation of a polypeptide of the invention comprising the following steps:

- a) culture of transformed cells under conditions allowing the expression of a recombinant polypeptide of nucleotide sequence according to the invention;
- b) if need be, recovery of said recombinant polypeptide.

When the procedure for preparation of a polypeptide of the invention employs a transgenic animal according to the invention, the recombinant polypeptide is then extracted from said animal.

The invention also relates to a polypeptide which is capable of being obtained by a procedure of the invention such as described previously.

The invention also comprises a procedure for preparation of a synthetic polypeptide, characterized



in that it uses a sequence of amino acids of polypeptides according to the invention.

The invention likewise relates to a synthetic polypeptide obtained by a procedure according to the  
5 invention.

The polypeptides according to the invention can likewise be prepared by techniques which are conventional in the field of the synthesis of peptides. This synthesis can be carried out in homogeneous  
10 solution or in solid phase.

For example, recourse can be made to the technique of synthesis in homogeneous solution described by Houben-Weyl in 1974.

This method of synthesis consists in  
15 successively condensing, two by two, the successive amino acids in the order required, or in condensing amino acids and fragments formed previously and already containing several amino acids in the appropriate order, or alternatively several fragments previously  
20 prepared in this way, it being understood that it will be necessary to protect beforehand all the reactive functions carried by these amino acids or fragments, with the exception of amine functions of one and carboxyls of the other or vice-versa, which must  
25 normally be involved in the formation of peptide bonds, especially after activation of the carboxyl function, according to the methods well known in the synthesis of peptides.

According to another preferred technique of the  
30 invention, recourse will be made to the technique described by Merrifield.

To make a peptide chain according to the Merrifield procedure, recourse is made to a very porous polymeric resin, on which is immobilized the first C-  
35 terminal amino acid of the chain. This amino acid is immobilized on a resin through its carboxyl group and its amine function is protected. The amino acids which are going to form the peptide chain are thus

immobilized, one after the other, on the amino group, which is deprotected beforehand each time, of the portion of the peptide chain already formed, and which is attached to the resin. When the whole of the desired peptide chain has been formed, the protective groups of the different amino acids forming the peptide chain are eliminated and the peptide is detached from the resin with the aid of an acid.

The invention additionally relates to hybrid polypeptides having at least one polypeptide according to the invention, and a sequence of a polypeptide capable of inducing an immune response in man or animals.

Advantageously, the antigenic determinant is such that it is capable of inducing a humoral and/or cellular response.

It will be possible for such a determinant to comprise a polypeptide according to the invention in glycosylated form used with a view to obtaining immunogenic compositions capable of inducing the synthesis of antibodies directed against multiple epitopes. Said polypeptides or their glycosylated fragments are likewise part of the invention.

These hybrid molecules can be formed, in part, of a polypeptide carrier molecule or of fragments thereof according to the invention, associated with a possibly immunogenic part, in particular an epitope of the diphtheria toxin, the tetanus toxin, a surface antigen of the hepatitis B virus (patent FR 79 21811), the VP1 antigen of the poliomyelitis virus or any other viral or bacterial toxin or antigen.

The procedures for synthesis of hybrid molecules encompass the methods used in genetic engineering for constructing hybrid nucleotide sequences coding for the polypeptide sequences sought. It will be possible, for example, to refer advantageously to the technique for obtainment of genes coding for fusion proteins described by Minton in 1984.

Said hybrid nucleotide sequences coding for a hybrid polypeptide as well as the hybrid polypeptides according to the invention characterized in that they are recombinant polypeptides obtained by the expression  
5 of said hybrid nucleotide sequences are likewise part of the invention.

The invention likewise comprises the vectors characterized in that they contain one of said hybrid nucleotide sequences. The host cells transformed by  
10 said vectors, the transgenic animals comprising one of said transformed cells as well as the procedures for preparation of recombinant polypeptides using said vectors, said transformed cells and/or said transgenic animals are, of course, likewise part of the invention.

15 The polypeptides according to the invention, the antibodies according to the invention described below and the nucleotide sequences according to the invention can advantageously be employed in procedures for the detection and/or identification of PWD  
20 circovirus, or of porcine circovirus other than a PWD circovirus, in a biological sample (biological tissue or fluid) capable of containing them. These procedures, according to the specificity of the polypeptides, the antibodies and the nucleotide sequences according to  
25 the invention which will be used, will in particular be able to detect and/or to identify a PWD circovirus or a porcine circovirus other than a PWD circovirus or other than the PWD circovirus of type B.

The polypeptides according to the invention can  
30 advantageously be employed in a procedure for the detection and/or the identification of PWD circovirus of type A, of type B, of type A or B, or porcine circovirus other than the PWD circovirus of type B, or of porcine circovirus other than the PWD circovirus of  
35 type A or B, in a biological sample (biological tissue or fluid) capable of containing them, characterized in that it comprises the following steps:

- a) contacting of this biological sample with a polypeptide or one of its fragments according to the invention (under conditions allowing an immunological reaction between said polypeptide and the antibodies possibly present in the biological sample);
- b) demonstration of the antigen-antibody complexes possibly formed.

In the present description, PWD circovirus, except if a particular mention is indicated, will be understood as designating a PWD circovirus of type A or of type B, and porcine circovirus other than PWD, except if a particular mention is indicated, will be understood as designating a porcine circovirus other than a PWD circovirus of type A and B.

Preferably, the biological sample is formed by a fluid, for example a pig serum, whole blood or biopsies.

Any conventional procedure can be employed for carrying out such a detection of the antigen-antibody complexes possibly formed.

By way of example, a preferred method brings into play immunoenzymatic processes according to the ELISA technique, by immunofluorescence, or radioimmunological processes (RIA) or their equivalent.

Thus, the invention likewise relates to the polypeptides according to the invention, labeled with the aid of an adequate label such as of the enzymatic, fluorescent or radioactive type.

Such methods comprise, for example, the following steps:

- deposition of determined quantities of a polypeptide composition according to the invention in the wells of a microtiter plate,
- introduction into said wells of increasing dilutions of serum, or of a biological sample other than that defined previously, having to be analyzed,

- incubation of the microplate,
- introduction into the wells of the microtiter plate of labeled antibodies directed against pig immunoglobulins, the labeling of these antibodies having been carried out with the aid of an enzyme selected from those which are capable of hydrolyzing a substrate by modifying the absorption of the radiation of the latter, at least at a determined wavelength, for example at 550 nm,
- detection, by comparison with a control test, of the quantity of hydrolyzed substrate.

The invention likewise relates to a kit or set for the detection and/or identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:

- a polypeptide according to the invention,
- if need be, the reagents for the formation of the medium favorable to the immunological or specific reaction,
- if need be, the reagents allowing the detection of the antigen-antibody complexes produced by the immunological reaction between the polypeptide(s) of the invention and the antibodies possibly present in the biological sample, these reagents likewise being able to carry a label, or to be recognized in their turn by a labeled reagent, more particularly in the case where the polypeptide according to the invention is not labeled,
- if need be, a biological reference sample (negative control) devoid of antibodies recognized by a polypeptide according to the invention,
- if need be, a biological reference sample (positive control) containing a predetermined quantity of antibodies recognized by a polypeptide according to the invention.

The polypeptides according to the invention allow monoclonal or polyclonal antibodies to be prepared which are characterized in that they specifically recognize the polypeptides according to the invention. It will advantageously be possible to prepare the monoclonal antibodies from hybridomas according to the technique described by Kohler and Milstein in 1975. It will be possible to prepare the polyclonal antibodies, for example, by immunization of an animal, in particular a mouse, with a polypeptide or a DNA, according to the invention, associated with an adjuvant of the immune response, and then purification of the specific antibodies contained in the serum of the immunized animals on an affinity column on which the polypeptide which has served as an antigen has previously been immobilized. The polyclonal antibodies according to the invention can also be prepared by purification, on an affinity column on which a polypeptide according to the invention has previously been immobilized, of the antibodies contained in the serum of pigs infected by a PWD circovirus.

The invention likewise relates to mono- or polyclonal antibodies or their fragments, or chimeric antibodies, characterized in that they are capable of specifically recognizing a polypeptide according to the invention.

It will likewise be possible for the antibodies of the invention to be labeled in the same manner as described previously for the nucleic probes of the invention, such as a labeling of enzymatic, fluorescent or radioactive type.

The invention is additionally directed at a procedure for the detection and/or identification of PWD circovirus, of porcine circovirus other than a PWD circovirus, or other than the PWD circovirus of type B, in a biological sample, characterized in that it comprises the following steps:

a) contacting of the biological sample (biological tissue or fluid) with a mono- or polyclonal antibody according to the invention (under conditions allowing an immunological reaction between said antibodies and the polypeptides of PWD circovirus, of porcine circovirus other than a PWD circovirus, of porcine circovirus other than the PWD circovirus of type B, possibly present in the biological sample);

b) demonstration of the antigen-antibody complex possibly formed.

Likewise within the scope of the invention is a kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following components:

- a polyclonal or monoclonal antibody according to the invention, if need be labeled;
- if need be, a reagent for the formation of the medium favorable to the carrying out of the immunological reaction;
- if need be, a reagent allowing the detection of the antigen-antibody complexes produced by the immunological reaction, this reagent likewise being able to carry a label, or being capable of being recognized in its turn by a labeled reagent, more particularly in the case where said monoclonal or polyclonal antibody is not labeled;
- if need be, reagents for carrying out the lysis of cells of the sample tested.

The present invention likewise relates to a procedure for the detection and/or the identification of PWD, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, in a biological sample, characterized in that it employs a nucleotide sequence according to the invention.



More particularly, the invention relates to a procedure for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, in a biological sample, characterized in that it contains the following steps:

a) if need be, isolation of the DNA from the biological sample to be analyzed;

b) specific amplification of the DNA of the sample with the aid of at least one primer, or a pair of primers, according to the invention;

c) demonstration of the amplification products.

These can be detected, for example, by the technique of molecular hybridization utilizing a nucleic probe according to the invention. This probe will advantageously be labeled with a nonradioactive (cold probe) or radioactive element.

For the purposes of the present invention, "DNA of the biological sample" or "DNA contained in the biological sample" will be understood as meaning either the DNA present in the biological sample considered, or possibly the cDNA obtained after the action of an enzyme of reverse transcriptase type on the RNA present in said biological sample.

Another aim of the present invention consists in a procedure according to the invention, characterized in that it comprises the following steps:

a) contacting of a nucleotide probe according to the invention with a biological sample, the DNA contained in the biological sample having, if need be, previously been made accessible to hybridization under conditions allowing the hybridization of the probe with the DNA of the sample;

b) demonstration of the hybrid formed between the nucleotide probe and the DNA of the biological sample.

The present invention also relates to a procedure according to the invention, characterized in that it comprises the following steps:

a) contacting of a nucleotide probe immobilized on a support according to the invention with a biological sample, the DNA of the sample having, if need be, previously been made accessible to hybridization, under  
5 conditions allowing the hybridization of the probe with the DNA of the sample;

b) contacting of the hybrid formed between the nucleotide probe immobilized on a support and the DNA contained in the biological sample, if need be after  
10 elimination of the DNA of the biological sample which has not hybridized with the probe, with a nucleotide probe labeled according to the invention;

c) demonstration of the novel hybrid formed in step b).

15 According to an advantageous embodiment of the procedure for detection and/or identification defined previously, this is characterized in that, prior to step a), the DNA of the biological sample is first amplified with the aid of at least one primer according  
20 to the invention.

The invention is additionally directed at a kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than the PWD circovirus or of porcine circovirus other than the  
25 PWD circovirus of type B, characterized in that it comprises the following elements:

a) a nucleotide probe according to the invention;  
b) if need be, the reagents necessary for the carrying out of a hybridization reaction;  
30 c) if need be, at least one primer according to the invention as well as the reagents necessary for an amplification reaction of the DNA.

The invention likewise relates to a kit or set for the detection and/or the identification of PWD  
35 circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following components:

a) a nucleotide probe, called a capture probe, according to the invention;

b) an oligonucleotide probe, called a revealing probe, according to the invention,

5 c) if need be, at least one primer according to the invention, as well as the reagents necessary for an amplification reaction of the DNA.

The invention also relates to a kit or set for the detection and/or identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of  
10 porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:

a) at least one primer according to the invention;

15 b) if need be, the reagents necessary for carrying out a DNA amplification reaction;

c) if need be, a component allowing the sequence of the amplified fragment to be verified, more particularly an oligonucleotide probe according to the  
20 invention.

The invention additionally relates to the use of a nucleotide sequence according to the invention, of a polypeptide according to the invention, of an antibody according to the invention, of a cell  
25 according to the invention, and/or of an animal transformed according to the invention, for the selection of an organic or inorganic compound capable of modulating, inducing or inhibiting the expression of genes, and/or of modifying the cellular replication of  
30 PWD circovirus or capable of inducing or of inhibiting the pathologies linked to an infection by a PWD circovirus.

The invention likewise comprises a method of selection of compounds capable of binding to a  
35 polypeptide or one of its fragments according to the invention, capable of binding to a nucleotide sequence according to the invention, or capable of recognizing an antibody according to the invention, and/or capable

of modulating, inducing or inhibiting the expression of genes, and/or of modifying the cellular replication of PWD circovirus or capable of inducing or inhibiting the pathologies linked to an infection by a PWD circovirus,  
5 characterized in that it comprises the following steps:

a) contacting of said compound with said polypeptide, said nucleotide sequence, or with a cell transformed according to the invention and/or administration of said compound to an animal  
10 transformed according to the invention;

b) determination of the capacity of said compound to bind to said polypeptide or said nucleotide sequence, or to modulate, induce or inhibit the expression of genes, or to modulate the growth or the  
15 replication of PWD circovirus, or to induce or inhibit in said transformed animal the pathologies linked to an infection by PWD circovirus (designated activity of said compound).

The compounds capable of being selected can be organic compounds such as polypeptides or carbohydrates or any other organic or inorganic compounds already known, or novel organic compounds elaborated by molecular modelling techniques and obtained by chemical or biochemical synthesis, these techniques being known  
20 to the person skilled in the art.

It will be possible to use said selected compounds to modulate the cellular replication of PWD circovirus and thus to control infection by this virus, the methods allowing said modulations to be determined  
30 being well known to the person skilled in the art.

This modulation can be carried out, for example, by an agent capable of binding to a protein and thus of inhibiting or of potentiating its biological activity, or capable of binding to an  
35 envelope protein of the external surface of said virus and of blocking the penetration of said virus into the host cell or of favoring the action of the immune system of the infected organism directed against said

virus. This modulation can likewise be carried out by an agent capable of binding to a nucleotide sequence of a DNA of said virus and of blocking, for example, the expression of a polypeptide whose biological or structural activity is necessary for the replication or for the proliferation of said virus host cells to host cells in the host animal.

The invention relates to the compounds capable of being selected by a selection method according to the invention.

The invention likewise relates to a pharmaceutical composition comprising a compound selected from the following compounds:

- a) a nucleotide sequence according to the invention;
- b) a polypeptide according to the invention;
- c) a vector, a viral particle or a cell transformed according to the invention;
- d) an antibody according to the invention;
- e) a compound capable of being selected by a selection method according to the invention; possibly in combination with a pharmaceutically acceptable vehicle and, if need be, with one or more adjuvants of the appropriate immunity.

The invention also relates to an immunogenic and/or vaccine composition, characterized in that it comprises a compound selected from the following compounds:

- a) a nucleotide sequence according to the invention;
- b) a polypeptide according to the invention;
- c) a vector or a viral particle according to the invention; and
- d) a cell according to the invention.

The invention additionally relates to a vaccine composition according to the invention, characterized in that it comprises a mixture of at least two of said compounds a), b), c) and d) above and in that one of

the two said compounds is related to the PWD circovirus of type A and the other is related to the PWD circovirus of type B.

5 A compound related to the PWD circovirus of type A or of type B is understood here as respectively designating a compound obtained from the genomic sequence of the PWD circovirus of type A or of type B.

10 The invention is additionally aimed at an immunogenic and/or vaccine composition, characterized in that it comprises at least one of the following compounds:

- a nucleotide sequence SEQ ID No. 11, SEQ ID No. 12, or one of their fragments;
- a polypeptide of sequence SEQ ID No. 14, SEQ ID 15 No. 15, or one of their fragments;
- a vector or a viral particle comprising a nucleotide sequence SEQ ID No. 11, SEQ ID No. 12, or one of their fragments;
- a transformed cell capable of expressing a 20 polypeptide of sequence SEQ ID No. 14, SEQ ID No. 15, or one of their fragments; or
- a mixture of at least two of said compounds.

25 The invention also comprises an immunogenic and/or vaccine composition according to the invention, characterized in that it comprises said mixture of at least two of said compounds as a combination product for simultaneous, separate or protracted use for the prevention or the treatment of infection by a PWD circovirus, especially of type B.

30 In a preferred embodiment, the vaccine composition according to the invention comprises the mixture of the following compounds:

- a pcDNA3 plasmid containing a nucleic acid of sequence SEQ ID No. 11;
- 35 - a pcDNA3 plasmid containing a nucleic acid of sequence SEQ ID No. 12;
- a pcDNA3 plasmid containing a nucleic acid coding for the GM-CSF protein;

- a recombinant baculovirus containing a nucleic acid of sequence SEQ ID No. 11;

- a recombinant baculovirus containing a nucleic acid of sequence SEQ ID No. 12; and

5       - if need be, an adjuvant of the appropriate immunity, especially the adjuvant AIF<sup>TM</sup>.

The invention is likewise directed at a pharmaceutical composition according to the invention, for the prevention or the treatment of an infection by  
10 a PWD circovirus.

The invention is also directed at a pharmaceutical composition according to the invention for the prevention or the treatment of an infection by the PWD circovirus of type B.

15       The invention likewise concerns the use of a composition according to the invention, for the preparation of a medicament intended for the prevention or the treatment of infection by a PWD circovirus, preferably by the PWD circovirus of type B.

20       Under another aspect, the invention relates to a vector, a viral particle or a cell according to the invention, for the treatment and/or the prevention of a disease by gene therapy.

Finally, the invention comprises the use of a  
25 vector, of a viral particle or of a cell according to the invention for the preparation of a medicament intended for the treatment and/or the prevention of a disease by gene therapy.

The polypeptides of the invention entering into  
30 the immunogenic or vaccine compositions according to the invention can be selected by techniques known to the person skilled in the art such as, for example, depending on the capacity of said polypeptides to stimulate the T cells, which is translated, for  
35 example, by their proliferation or the secretion of interleukins, and which leads to the production of antibodies directed against said polypeptides.



In pigs, as in mice, in which a weight dose of the vaccine composition comparable to the dose used in man is administered, the antibody reaction is tested by taking of the serum followed by a study of the formation of a complex between the antibodies present in the serum and the antigen of the vaccine composition, according to the usual techniques.

The pharmaceutical compositions according to the invention will contain an effective quantity of the compounds of the invention, that is to say in sufficient quantity of said compound(s) allowing the desired effect to be obtained, such as, for example, the modulation of the cellular replication of PWD circovirus. The person skilled in the art will know how to determine this quantity, as a function, for example, of the age and of the weight of the individual to be treated, of the state of advancement of the pathology, of the possible secondary effects and by means of a test of evaluation of the effects obtained on a population range, these tests being known in these fields of application.

According to the invention, said vaccine combinations will preferably be combined with a pharmaceutically acceptable vehicle and, if need be, with one or more adjuvants of the appropriate immunity.

Today, various types of vaccines are available for protecting animals or man against infectious diseases: attenuated living microorganisms (*M. bovis* - BCG for tuberculosis), inactivated microorganisms (influenza virus), acellular extracts (*Bordetella pertussis* for whooping cough), recombined proteins (surface antigen of the hepatitis B virus), polysaccharides (pneumococcal). Vaccines prepared from synthetic peptides or genetically modified microorganisms expressing heterologous antigens are in the course of experimentation. More recently still, recombined plasmid DNAs carrying genes coding for protective antigens have been proposed as an

alternative vaccine strategy. This type of vaccination is carried out with a particular plasmid originating from a plasmid of *E.coli* which does not replicate *in vivo* and which codes uniquely for the vaccinating protein. Animals have been immunized by simply injecting the naked plasmid DNA into the muscle. This technique leads to the expression of the vaccine protein *in situ* and to an immune response of cellular type (CTL) and of humoral type (antibody). This double induction of the immune response is one of the principal advantages of the vaccination technique with naked DNA.

The vaccine compositions comprising nucleotide sequences or vectors into which are inserted said sequences are especially described in the international application No. WO 90/11092 and likewise in the international application No. WO 95/11307.

The constitutive nucleotide sequence of the vaccine composition according to the invention can be injected into the host after having been coupled to compounds which favor the penetration of this polynucleotide into the interior of the cell or its transport to the cell nucleus. The resultant conjugates can be encapsulated in polymeric microparticles, as described in the international application No. WO 94/27238 (Medisorb Technologies International).

According to another embodiment of the vaccine composition according to the invention, the nucleotide sequence, preferably a DNA, is complexed with DEAE-dextran (Pagano et al., 1967) or with nuclear proteins (Kaneda et al., 1989), with lipids (Felgner et al., 1987) or encapsulated in liposomes (Fraley et al., 1980) or else introduced in the form of a gel facilitating its transfection into the cells (Midoux et al., 1993, Pastore et al., 1994). The polynucleotide or the vector according to the invention can also be in suspension in a buffer solution or be combined with liposomes.

Advantageously, such a vaccine will be prepared according to the technique described by Tacson et al. or Huygen et al. in 1996 or alternatively according to the technique described by Davis et al. in the international application No. WO 95/11307.

Such a vaccine can likewise be prepared in the form of a composition containing a vector according to the invention, placed under the control of regulation elements allowing its expression in man or animal. It will be possible, for example, to use, by way of in vivo expression vector of the polypeptide antigen of interest, the plasmid pcDNA3 or the plasmid pcDNA1/neo, both marketed by Invitrogen (R&D Systems, Abingdon, United Kingdom). It is also possible to use the plasmid V1Jns.tPA, described by Shiver et al. in 1995. Such a vaccine will advantageously comprise, apart from the recombinant vector, a saline solution, for example a sodium chloride solution.

Pharmaceutically acceptable vehicle is understood as designating a compound or a combination of compounds entering into a pharmaceutical composition or vaccine which does not provoke secondary reactions and which allows, for example, the facilitation of the administration of the active compound, an increase in its duration of life and/or its efficacy in the body, an increase in its solubility in solution or alternatively an improvement in its conservation. These pharmaceutically acceptable vehicles are well known and will be adapted by the person skilled in the art as a function of the nature and of the mode of administration of the chosen active compound.

As far as the vaccine formulations are concerned, these can comprise adjuvants of the appropriate immunity which are known to the person skilled in the art, such as, for example, aluminum hydroxide, a representative of the family of muramyl peptides such as one of the peptide derivatives of N-

acetyl muramyl, a bacterial lysate, or alternatively Freund's incomplete adjuvant.

These compounds can be administered by the systemic route, in particular by the intravenous route, 5 by the intramuscular, intradermal or subcutaneous route, or by the oral route. In a more preferred manner, the vaccine composition comprising polypeptides according to the invention will be administered by the intramuscular route, through the food or by 10 nebulization several times, staggered over time.

Their administration modes, dosages and optimum pharmaceutical forms can be determined according to the criteria generally taken into account in the establishment of a treatment adapted to an animal such 15 as, for example, the age or the weight, the seriousness of its general condition, the tolerance to the treatment and the secondary effects noted.

The present invention likewise relates to the use of nucleotide sequences of PWD circovirus according 20 to the invention for the construction of autoreplicative retroviral vectors and the therapeutic applications of these, especially in the field of human gene therapy in vivo.

The feasibility of gene therapy applied to man 25 no longer needs to be demonstrated and this relates to numerous therapeutic applications like genetic diseases, infectious diseases and cancers. Numerous documents of the prior art describe the means of employing gene therapy, especially through viral 30 vectors. Generally speaking, the vectors are obtained by deletion of at least some of the viral genes which are replaced by the genes of therapeutic interest. Such vectors can be propagated in a complementation line which supplies in trans the deleted viral functions in 35 order to generate a defective viral vector particle for replication but capable of infecting a host cell. To date, the retroviral vectors are amongst the most widely used and their mode of infection is widely

described in the literature accessible to the person skilled in the art.

The principle of gene therapy is to deliver a functional gene, called a gene of interest, of which  
5 the RNA or the corresponding protein will produce the desired biochemical effect in the targeted cells or tissues. On the one hand, the insertion of genes allows the prolonged expression of complex and unstable molecules such as RNAs or proteins which can be  
10 extremely difficult or even impossible to obtain or to administer directly. On the other hand, the controlled insertion of the desired gene into the interior of targeted specific cells allows the expression product to be regulated in defined tissues. For this, it is  
15 necessary to be able to insert the desired therapeutic gene into the interior of chosen cells and thus to have available a method of insertion capable of specifically targeting the cells or the tissues chosen.

Among the methods of insertion of genes, such  
20 as, for example, microinjection, especially the injection of naked plasmid DNA (Derse, D. et al., 1995, and Zhao, T.M. et al., 1996), electroporation, homologous recombination, the use of viral particles, such as retroviruses, is widespread. However, applied  
25 in vivo, the gene transfer systems of recombinant retroviral type at the same time have a weak infectious power (insufficient concentration of viral particles) and a lack of specificity with regard to chosen target cells.

30 The production of cell-specific viral vectors, having a tissue-specific tropism, and whose gene of interest can be translated adequately by the target cells, is realizable, for example, by fusing a specific ligand of the target host cells to the N-terminal part  
35 of a surface protein of the envelope of PWD circovirus. It is possible to mention, for example, the construction of retroviral particles having the CD4 molecule on the surface of the envelope so as to target

the human cells infected by the HIV virus (YOUNG, J.A.T. et al., Sciences 1990, 250, 1421-1423), viral particles having a peptide hormone fused to an envelope protein to specifically infect the cells expressing the  
5 corresponding receptor (KASAHARA, N. et al., Sciences 1994, 266, 1373-1376) or else alternatively viral particles having a fused polypeptide capable of immobilizing on the receptor of the epidermal growth factor (EGF) (COSSET, F.L. et al., J. of Virology 1995,  
10 69, 10, 6314-6322). In another approach, single-chain fragments of antibodies directed against surface antigens of the target cells are inserted by fusion with the N-terminal part of the envelope protein (VALSESIA-WITTMAN, S. et al., J. of Virology 1996, 70,  
15 3, 2059-2064; TEARINA CHU, T.H. et al., J. of Virology 1997, 71, 1, 720-725).

For the purposes of the present invention, a gene of interest in use in the invention can be obtained from a eukaryotic or prokaryotic organism or  
20 from a virus by any conventional technique. It is, preferably, capable of producing an expression product having a therapeutic effect and it can be a product homologous to the cell host or, alternatively, heterologous. In the scope of the present invention, a  
25 gene of interest can code for an (i) intracellular or (ii) membrane product present on the surface of the host cell or (iii) secreted outside the host cell. It can therefore comprise appropriate additional elements such as, for example, a sequence coding for a secretion  
30 signal. These signals are known to the person skilled in the art.

In accordance with the aims pursued by the present invention, a gene of interest can code for a protein corresponding to all or part of a native  
35 protein as found in nature. It can likewise be a chimeric protein, for example arising from the fusion of polypeptides of various origins or from a mutant having improved and/or modified biological properties.



Such a mutant can be obtained, by conventional biological techniques, by substitution, deletion and/or addition of one or more amino acid residues.

It is very particularly preferred to employ a gene of therapeutic interest coding for an expression product capable of inhibiting or retarding the establishment and/or the development of a genetic or acquired disease. A vector according to the invention is in particular intended for the prevention or for the treatment of cystic fibrosis, of hemophilia A or B, of Duchenne's or Becker's myopathy, of cancer, of AIDS and of other bacteria or infectious diseases due to a pathogenic organism: virus, bacteria, parasite or prion. The genes of interest utilizable in the present invention are those which code, for example, for the following proteins:

- a cytokine and especially an interleukin, an interferon, a tissue necrosis factor and a growth factor and especially a hematopoietic growth factor (G-CSF, GM-CSF),
- a factor or cofactor involved in clotting and especially factor VIII, von Willebrand's factor, antithrombin III, protein C, thrombin and hirudin,
- an enzyme or an enzyme inhibitor such as the inhibitors of viral proteases,
- an expression product of a suicide gene such as thymidine kinase of the HSV virus (herpesvirus) of type 1,
- an activator or an inhibitor of ion channels,
- a protein of which the absence, the modification or the deregulation of expression is responsible for a genetic disease, such as the CFTR protein, dystrophin or minidystrophin, insulin, ADA (adenosine diaminase), glucocerebrosidase and phenylhydroxylase,
- a protein capable of inhibiting the initiation or the progression of cancers, such as the expression



products of tumor suppressor genes, for example the P53 and Rb genes,

- a protein capable of stimulating an immune or an antibody response, and

5 - a protein capable of inhibiting a viral infection or its development, for example the antigenic epitopes of the virus in question or altered variants of viral proteins capable of entering into competition with the native viral proteins.

10 The invention thus relates to the vectors characterized in that they comprise a nucleotide sequence of PWD circovirus according to the invention, and in that they additionally comprise a gene of interest.

15 The present invention likewise relates to viral particles generated from said vector according to the invention. It additionally relates to methods for the preparation of viral particles according to the invention, characterized in that they employ a vector  
20 according to the invention, including viral pseudo-particles (VLP, virus-like particles).

The invention likewise relates to animal cells transfected by a vector according to the invention.

Likewise comprised in the invention are animal  
25 cells, especially mammalian, infected by a viral particle according to the invention.

The present invention likewise relates to a vector, a viral particle or a cell according to the invention, for the treatment and/or the prevention of a  
30 genetic disease or of an acquired disease such as cancer or an infectious disease. The invention is likewise directed at a pharmaceutical composition comprising, by way of therapeutic or prophylactic agent, a vector or a cell according to the invention,  
35 in combination with a vehicle acceptable from a pharmaceutical point of view.

Other characteristics and advantages of the invention appear in the examples and the following figures:

Legends to the figures:

- 5 Figure 1: Experimental scheme which has made it possible to bring about the isolation and the identification of the circovirus associated with PWD of type A and B.

10 Test 1: experimental reproduction of the PWD by inoculation of pig organ homogenates from farms affected by PWD.

Test 2: experimental reproduction of PWD.

Test 3: experimental reproduction of PWD.

Test 4: no experimental reproduction of PWD.

- 15 Figure 2: Organization of the genome of the circovirus associated with PWD of type A (PCVA)

- strand of (+) polarity (SEQ ID No. 1);
- strand of (-) polarity (SEQ ID No. 2, represented according to the orientation 3' → 5');
- 20 - sequences of amino acids of proteins encoded by the two DNA strands in the three possible reading frames.

Figure 3: Alignment of the nucleotide sequence SEQ ID No. 1 of the PWD circovirus of type A (PCVA) and of the  
25 MEEHAN strain and MANKERTZ strain circoviruses of the porcine cell lines.

Figure 4: Alignment of the sequence of amino acids SEQ ID No. 6 of a polypeptide encoded by the nucleotide sequence SEQ ID No. 3 (ORF1) of the PWD circovirus of  
30 type A (PCVA) and of corresponding nucleotide sequences of the MEEHAN strain and MANKERTZ strain circoviruses of the porcine cell lines.

Figure 5: Alignment of the sequence of amino acids SEQ ID No. 7 of a polypeptide encoded by the nucleotide  
35 sequence SEQ ID No. 4 (ORF2) of the PWD circovirus of type A (PCVA) and of corresponding nucleotide sequences of the MEEHAN strain and MANKERTZ strain circoviruses of the porcine cell lines.

Figure 6: Alignment of the sequence of amino acids SEQ ID No. 8 of a polypeptide encoded by the nucleotide sequence SEQ ID No. 5 (ORF3) of the PWD circovirus of type A (PCVA) and of corresponding nucleotide sequences of the MEEHAN strain and MANKERTZ strain circoviruses of the porcine cell lines.

Figure 7: Western blot analysis of recombinant proteins of the PWD circovirus of type A (PCVA).

The analyses were carried out on cell extracts of Sf9 cells obtained after infection with recombinant baculovirus PCF ORF 1.

Figure 8: Organization of the genome of the circovirus associated with the PWD of type B (PCVB)

- strand of (+) polarity (SEQ ID No. 9);
- strand of (-) polarity (SEQ ID No. 10, represented according to the orientation 3' → 5');
- sequence of amino acids of proteins encoded by the two DNA strands in the three possible reading frames.

Figure 9: Evolution of the daily mean gain (DMG) of pig farms affected by piglet weight loss disease (PWD), placed under experimental conditions.

Figure 10: DMG compared for the 3 batches of pigs (F1, F3 and F4) calculated over a period of 28 days, after vaccination test.

Figure 11: Hyperthermia greater than 41°C, expressed as a percentage compared for the 3 batches of pigs (F1, F3 and F4) calculated per week over a period of 28 days, after vaccination test.

Figure 12: Membranes of peptide spots corresponding to the ORF2s revealed with the aid of an infected pig serum, originating from a conventional farm.

The numbers of specific peptides of the circovirus of type B as well as their nonreactive homologs (type A) are indicated in bold.

The nonspecific immunogenic peptides are indicated in italics.

Figure 13: Alignment of amino acid sequences of proteins encoded by the ORF2 of the PWD circovirus of type A and by the ORF'2 of the PWD circovirus of type B. The position of 4 peptides corresponding to specific epitopes of the PWD circovirus of type B is indicated on the corresponding sequence by a bold line, their homolog on the sequence of the PWD circovirus of type A is likewise indicated by an ordinary line.

## 10 EXAMPLES

EXAMPLE 1: Cloning, sequencing and characterization of the PWD circovirus of type A (PCVA)

### 1 - Experimental procedures

Experimental reproduction of the infection and its syndrome (cf. Figure 1).

A first test was carried out with pigs from a very well-kept farm, but affected by piglet weight loss disease (PWD), likewise called fatal piglet wasting (FPW). Tests carried out with SPF (specific pathogen-free) pigs showed a transfer of contaminant(s) finding expression in a complex pathology combining hyperthermia, retardation of growth, diarrhea and conjunctivitis. The PDRS (porcine dysgenic and respiratory syndrome) virus, an infectious disease due to an arteriovirus) was rapidly isolated from breeding pigs and contact pigs. It should have been possible to attribute all the clinical signs to the presence of the PDRS virus. However, two farm pigs presented signs of FPW without the PDRS virus being isolated. The histological analyses and blood formulas, however, showed that these pigs were suffering from an infectious process of viral origin.

In a second test, 8-week SPF pigs were inoculated by the intratracheal route with organ homogenates of two farm pigs suffering from FPW. The inoculated pigs exhibited hyperthermia 8 to 9 days post-infection, then their growth was retarded. Other SPF pigs, placed in contact, had similar, attenuated

signs 30 days after the initial experiment. No seroconversion with respect to a European or Canadian strain of PDRS virus was recorded in these animals.

5 A third test allowed the syndrome to be reproduced from samples taken from the pigs of the second test.

#### Conclusion

10 The syndrome is reproduced under the experimental conditions. It is determined by at least one infectious agent, which is transmittable by direct contact. The clinical constants are a sometimes high hyperthermia (greater than or equal to 41.5°C) which develops 8 to 10 days after infection. Retardation of the growth can be observed. The other signs are a  
15 reversal of the blood formula (reversal of the lymphocyte/polynuclear ratio from 70/30 to 30/70) and frequent lesions on the ganglia, especially those draining the respiratory apparatus (ganglionic hypertrophy, loss of structure with necrosis and  
20 infiltration by mononucleated or plurinucleated giant cells).

#### 2 - Laboratory studies

Various cell supports including primary pig kidney cells or cell lines, pig testicle cells, monkey  
25 kidney cells, pig lymphocytes, pig alveolar macrophages and circulating blood monocytes were used to demonstrate the possible presence of a virus. No cytopathic effect was demonstrated in these cells. On the other hand, the use of a serum of a pig sick after  
30 experimental infection allowed an intracellular antigen to be revealed in the monocytes, the macrophages and approximately 10% of pig kidney (PK) cells infected with organ homogenates. This indirect revealing was carried out kinetically at different culture times. It  
35 is evident from this that the antigen initially appears in the nucleus of the infected cells before spreading into the cytoplasm. The successive passages in cell culture did not allow the signal to be amplified.

Under electron microscopy on organ homogenates, spherical particles labeled specifically by the serum of sick pigs, infected under the experimental conditions, were visualized. The size of these particles is estimated at 20 nm.

After two passages of these organ homogenates over pig lymphocytes and then three passages over pig kidney or testicle cells, a cytopathic effect developed and was amplified. An adenovirus was visualized in the electron microscope, which, under the experimental conditions, did not reproduce FPW (only a hyperthermia peak was noted 24 to 48 hours after infection, and then nothing more).

It has been possible to demonstrate DNA bands in certain samples of pigs infected under the experimental conditions and having exhibited signs of the disease (results not shown). A certain connection exists between the samples giving a positive result in cell culture and those having a DNA band.

#### Conclusion

At least two types of virus were demonstrated in the organ homogenates from pigs suffering from FPW. One is an adenovirus, but by itself alone it does not reproduce the disease. The other type of virus is a circovirus and is associated with FPW. This circovirus, of which two types have been isolated and sequenced, designated below PWD circovirus type A (or PCVA) and PWD circovirus of type B (or PCVB) have mutations with respect to the known sequences of circovirus which are nonpathogenic for the pig.

#### 3 - Cloning and sequencing of the DNA of the PWD circovirus of type A

Extraction of the replicative form (RF) DNA, cleavage by the Kpn I enzyme and amplification by a pair of primers flanking the Kpn I restriction site. Sequencing of the two strands at least twice by the Sanger method.

The nucleic sequence of the strand of (+) polarity of the genome of the PWD circovirus of type A (or PCVA), strain FPW, is represented by the sequence SEQ ID No. 1 in the list of sequences, the nucleic  
5 sequence of the strand of (-) polarity of the genome of the PWD circovirus of type A (or PCVA) being represented by the nucleic sequence 3' → 5' of Figure 3 or by the sequence SEQ ID No. 2 (represented according to the orientation 5' → 3') in the list of  
10 sequences.

The amino acid sequences SEQ ID No. 6, SEQ ID No. 7 and SEQ ID No. 8 of the list of sequences respectively represent the sequences of proteins encoded by the nucleic sequences of the 3 open reading  
15 frames SEQ ID No. 3 (ORF1), corresponding to the REP protein, SEQ ID No. 4 (ORF2) and SEQ ID No. 5 (ORF3), determined from the sequence SEQ ID No. 1 of the strand of (+) polarity or of the nucleic sequence SEQ ID No. 2 of the strand of (-) polarity of the genome of the PWD  
20 circovirus of type A.

4 - Comparison of the nucleotide sequences and amino acids of the PWD circovirus of type A (or associated with PWD) which are obtained with the corresponding sequences of MEEHAN and MANKERTZ circoviruses of porcine cell lines  
25 Use of the DNA sequence analysis software, DNASIS.

Sequences of oligonucleotides used as primers or probes in the detection and/or identification procedures  
30 1. specific detection of the PWD circovirus of type A:  
primer PCV 5: 5' GTG TGC TCG ACA TTG GTG TG 3';  
primer PCV 10: 5' TGG AAT GTT AAC GAG CTG AG 3';  
2. specific detection of the circovirus of the  
35 cell lines:  
primer PCF 5: 5' GTG TGC TCG ACA TTG GTG TG 3';  
primer MEE 1: 5' TGG AAT GTT AAC TAC CTC AA 3';  
3. differential detection:



the pairs of primers used are those described, for example, in the paragraphs 1 and 2 above;

4. detection of the monomeric circular replicative forms RF:

5 primer PCV 5: 5' GTG TGC TCG ACA TTG GTG TG 3';  
primer PCV 6: 5' CTC GCA GCC ATC TTG GAA TG 3';

5. detection of the vectors carrying the dimers in tandem:

Nar dimer:

10 primer KS 620: 5' CGC GCG TAA TAC GAC TCA CT 3';  
primer PCV 5: 5' GTG TGC TCG ACA TTG GTG TG 3';

Kpn dimer:

primer KS 620: 5' CGC GCG TAA TAC GAC TCA CT 3';  
primer PCV 6: 5' CTC GCA GCC ATC TTG GAA TG 3';

15 6. differential detection:

the pairs of primers used are those described, for example, in paragraphs 4 and 5 above.

The procedures using the pairs or primers described in paragraphs 4 and 5 are of particular  
20 interest for differentially detecting the circular monomeric forms of specific replicative forms of the virion or of the DNA in replication and the dimeric forms found in the so-called in-tandem molecular constructs.

25 The in-tandem constructs of the viral genome (dimers) such as the constructs used for the preparation of the pBS KS + tandem PCV Kpn I vector, deposited at the CNCM under the number I-1891, 3 July 1997 (E. coli transformed by said vector) are very  
30 interesting for their use in methods of production in sufficient quantity of an inoculum formed of DNA, intended for the virus production, this in the absence of a satisfactory virus production protocol in a cell system. These said methods of production using these  
35 in-tandem constructs of the viral genome will allow the virulence factors to be studied by mutation and by way of consequence will be able to be used for the production of a collection of viruses carrying the

mutations indicated in the construction of vectors which will have the appropriate tropism and virulence. These vectors with autoreplicative structure have the sought gene transfer properties, especially for their applications in gene therapy, and in vaccinology.

5 Western-blot analysis of recombinant proteins of the PWD circovirus of type A

The results were obtained using a specific antiserum of the PWD circovirus produced during test 1  
10 (cf. Figure 1).

Type of products analyzed.

The analyses were carried out on cell extracts of Sf9 cells obtained after infection by the recombinant baculovirus PCV ORF 1.

15 The culture of Sf9 cells was carried out in a 25 cm<sup>2</sup> Petri dish according to the standard culture methods for these cells. After centrifugation, the cell pellets are taken up with 300 µl of PBS buffer (phosphate saline buffer).

20 Electrophoresis (PAGE-SDS)

The electrophoresis is carried out on the cell extracts of Sf9 cells obtained previously on 5 samples (cf. Table 1 below) under the following conditions:

% polyacrylamide gel: 8%; conditions: denaturing

25 Voltage: 80 V; duration: 135 mn.

Table 1: Nature of the samples subjected to electrophoresis

Well No.	1	2	3	4	5
Sample applied	PM Rainbow	Raoul 24 h	Raoul 48 h	Raoul 72 h	Raoul 96 h
µl of sample	10	15	15	15	15
µl of Laemmli 4X	0	5	5	5	5

Legends to Table 1:

Laemmli 4X: loading buffer

PM Rainbow: molecular-weight markers (35, 52, 77, 107, 160 and 250 kD)

Raoul 24 h, 48 h, 72 h and 96 h: expression products of the ORF1 of the PWD circovirus of type A.

5 Western blot

After electrophoresis, the bands obtained in the different wells are transferred to nitrocellulose membrane for 1 h at 100 v in a TGM buffer (tris-glycine-methanol).

10 The Western blot is carried out under the following conditions:

1) Saturation with a solution containing 5% of skimmed milk; 0.05% of Tween 20 in a TBS 1X buffer (tris buffer saline) for 30 min.

15 2) 1st antibody:

10 ml of PWD anticircovirus antibody of type A are added diluted to 1/100, then the reaction mixture is incubated for one night at 4°C. Three washes of 10 min in TBS 1X are carried out.

20 3) 2nd antibody:

10 ml of pig rabbit P164 antibody anti-immunoglobulins, coupled to peroxidase (Dakopath) are added diluted to 1/100, then the reaction medium is incubated for 3 hours at 37°C. Three washes of 10 min in TBS 1X are carried out.

25 4) Visualization

The substrate 4-chloro-1-naphthol in the presence of oxygenated water is used for visualization.

Results

30 The results are shown in Figure 7.

Kinetics of appearance of antibodies specific for the REP recombinant protein of the PWD circovirus of type A expressed in baculovirus after infection of pigs by the PWD circovirus of type A (test 4, cf. Figure 1)

35

After infection of the pigs, a sample of serum of each of the infected pigs is taken at different periods expressed in the table by the date of taking

(carried out here in the same year) and is then analyzed by Western blot.

The visualization of the specific antibodies is carried out in the manner described previously.

5 The results obtained are shown by Table 2 below.

Table 2: Kinetics of appearance of specific antibodies

Sample	Pigs	10/6	16/06	23/06	01/07	08/07	15/07	21/07
A3	1						Neg.	
Control	2						Neg.	
B2	1	Neg.	Neg.	Neg.	+	+	++	+++
Infec.	2	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
RP+	3	Neg.	Neg.	Neg.	Neg.	+	+	+
	4	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	++

10 Legends to Table 2:

A3 control: uninfected control animals;

B2 Infec. RP+: animals infected with pig kidney (PK) cells containing the circovirus;

Neg.: negative;

15 +, ++, +++: intensity scale of the positive reaction;  
10/06, 16/06, 23/06, 01/07, 08/07, 15/07, 21/07: dates expressed in day/month on which the different withdrawals of serum were carried out.

20 EXAMPLE 2: Cloning, sequencing and characterization of the type B PWD circovirus (PCVB)

The techniques used for cloning, sequencing and characterization of the type B PWD circovirus (PCVB) are those used in Example 1 above for the type A PWD  
25 circovirus (PCVA).

The nucleic sequence of the strand of (+) polarity of the genome of the PWD circovirus of type B (or PCVB) is represented by the sequence SEQ ID No. 9 in the sequence listing, the nucleic sequence of the  
30 strand of (-) polarity of the genome of the PWD circovirus of type B (or PCVB) being represented by the

nucleic sequence 3' → 5' of Figure 8 or by the sequence SEQ ID No. 10 (represented according to the orientation 5' → 3') in the sequence listing.

The amino acid sequences SEQ ID No. 14, SEQ ID No. 15 and SEQ ID No. 16 of the sequence listing respectively represent the sequences of the proteins encoded by the nucleic sequences of the 3 open reading frames SEQ ID No. 11 (ORF'1), corresponding to the REP protein, SEQ ID No. 12 (ORF'2) and SEQ ID No. 13 (ORF'3), determined from the sequence SEQ ID No. 9 of the strand of (+) polarity or from the nucleic sequence SEQ ID No. 10 of the strand of (-) polarity of the genome of the PWD circovirus of type B.

EXAMPLE 3: Comparative analysis of nucleotide sequences (ORF1, ORF2 and genomic) and amino acid sequences encoded by the ORF1 and the ORF2 of the PWD circoviruses of type A (PCVA) and of type B (PCVB)

The results expressed in % of homology are shown in Tables 3 and 4 below.

Table 3: Compared analysis of the amino acid sequences

% homology	ORF1	ORF2
PCVA/PCVB	80.4	56.2

Table 4: Compared analysis of the nucleotide sequences

% homology	Genomic	ORF1	ORF2	The remainder
PCVA/PCVB	70.4	80.4	60.1	66.1

EXAMPLE 4: Observation of the disease and reproduction of the disease under experimental conditions

a) Test No. 1: Observation of the disease

The objective is to take breeding animals at the start of disease and to place them under experimental conditions to follow the progression of

the pathology and describe all the clinical signs thereof. This first test was carried out on 3 breeding pigs aged 10 weeks of which 2 were already ill (suffering from wasting), and on 3 other pigs aged 13 weeks, not having signs of disease. The clinical observation was spread over a period of 37 days. Two pigs of 10 weeks wasted rapidly (pigs 1 and 2, Figure 9) and had to be painlessly killed 5 and 6 days after their arrival. A single pig exhibited hyperthermia over 5 days and diarrhea. Two other pigs exhibited dyspnea and cough, of which one additionally had hyperthermia, greater than 41°C, for the two first days of its stay. Another pig had retarded growth in the second week (pig 6, Figure 9), without any other clinical sign being recorded. On the lesional level, 5 pigs out of 6 exhibited macroscopic lesions of gray pneumonia, the sixth exhibited cicatricial lesions on the lung.

b) Test No. 2: Reproduction of the disease from inocula prepared in farm pigs.

The two sick pigs in test 1 served to prepare inocula which were tested in test 2 on specific-pathogen-free (SPF) pigs. The SPF pigs were aged 9 weeks at the time of inoculation. The clinical and lesional results are shown in Table 5.







In this test, there was no wasting, at the very most a retardation of the growth in the second, third or fourth week after infection. These data illustrate that certain breeding conditions probably favor the expression of the disease.

c) Tests No. 3 to No. 7: Reproduction of the experimental tests

The increase in the number of the experimental tests on pigs had the mastering and better characterization of the experimental model as an objective. All of the results are presented in Table 5.

Under the experimental conditions, PWD is thus characterized by a long incubation, of 8 to 14 days, true hyperthermia over 2 to 8 days, a decrease in food consumption and a retardation of the increase in weight on the second, third or fourth week post-infection. The lesional table associated with this clinical expression includes, in the main, ganglionic hypertrophy and lesions of pneumonia.

#### Conclusion

The perfection of this experimental model allows the direct etiological role of the PWD circovirus in the disease to be indisputably demonstrated. In addition, this model is an indispensable tool for the understanding of pathogenic mechanisms and the study of future vaccine candidates.

EXAMPLE 5: Demonstration of the vaccine composition protective efficacy produced from nucleic fragments of PWD circovirus sequence

#### 1) Animals used for the study

Piglets having the PWD disease, reproduced under experimental conditions described in paragraph c) of Example 4, were used in a protocol for evaluating the vaccine composition efficacy, comprising nucleic fragments of PWD circovirus sequence.

2) Tested vaccine composition and vaccination protocol

a) Components used for the study

The plasmids were obtained from the pcDNA3  
5 plasmid of INVITROGENE

- pcDNA3ORF- plasmids

These plasmids are plasmids which do not carry a PWD circovirus nucleic acid insert and are used as a negative control plasmid.

10 - pcDNA3ORF1+ plasmid and pcDNA3ORF2+ plasmid

The pcDNA3ORF1+ and pcDNA3ORF2+ plasmids are plasmids which carry a nucleic acid insert of the sequence of the PWD circovirus of TYPE B, respectively an insert comprising the nucleic acid fragment SEQ ID  
15 No. 11 (ORF'1) coding for the Rep protein of sequence SEQ ID No. 14 and an insert comprising the nucleic acid fragment SEQ ID No. 12 (ORF'2) coding for the protein of sequence SEQ ID No. 15, probably corresponding to the capsid protein, these nucleic constructs comprising  
20 the ATG initiation codon of the coding sequence of the corresponding protein.

- GMCSF+ plasmid

GM-CSF (granulocyte/macrophage colony stimulating factor) is a cytokine which occurs in the  
25 development, the maturation and the activation of macrophages, granulocytes and dendritic cells which present an antigen. The beneficial contribution of the GM-CSF in vaccination is considered to be a cellular activation with, especially, the recruitment and the  
30 differentiation of cells which present an antigen.

This pcDNA3-GMCSF+ plasmid carries a nucleic acid insert coding for the granulocyte/macrophage colony stimulation factor, the GM-CSF protein.

The gene coding for this GM-CSF protein was  
35 cloned and sequenced by Inumaru et al. (Immunol. Cell Biol., 1995, 73 (5), 474-476). The pcDNA3-GMCSF+ plasmid was obtained by Dr. B. Charley of INRA of Jouy-en-Josas (78, France).

- Recombinant baculoviruses

The so-called ORF- baculoviruses are viruses not carrying any insert comprising a nucleic acid fragment capable of expressing a PWD circovirus protein.

The so-called ORF1+ (BAC ORF1+) or ORF2+ (BAC ORF2+) baculoviruses are recombinant baculoviruses respectively carrying an insert comprising a nucleic acid fragment SEQ ID No. 11 (ORF'1) and an insert comprising the nucleic acid fragment SEQ ID No. 12 (ORF'2).

- Adjuvant

The adjuvant supplied by the Seppic Company, a subsidiary of AIR LIQUIDE, is the adjuvant corresponding to the reference AIF SEPPIC.

b) Vaccination protocol

Weaned piglets aged 3 weeks are divided into four batches A, B, C and D each comprising 8 piglets.

Batches A, B and C, aged 3 weeks, each receive a first injection (injection M1) of 1 ml containing 200 micrograms of plasmids (naked DNA) in PBS, pH: 7.2, by the intramuscular route for each of the plasmids mentioned below for each batch, then, at the age of 5 weeks, a second injection (injection M2) comprising these same plasmids. A third injection is carried out simultaneously on the other side of the neck. This third injection comprises 1 ml of a suspension containing  $5.10^6$  cells infected by recombinant baculoviruses and 1 ml of AIF SEPPIC adjuvant.

Batch A (F1) (control batch):

- first injection

pcDNA3ORF1- plasmid, pcDNA3ORF2- plasmid and GMCSF+ plasmid.

- second and third injection (simultaneous)

pcDNA3ORF1- plasmid, pcDNA3ORF2- plasmid and GMCSF+ plasmid;

Cells transformed by baculoviruses not containing any nucleic acid insert coding for a PWD circovirus protein;  
AIF SEPPIC adjuvant.

5 Batch B (F2) (control batch):

- first injection

pcDNA3ORF1- plasmid, pcDNA3ORF2- plasmid and GMCSF+ plasmid;

- second and third injection (simultaneous)

10 pcDNA3ORF1- plasmid, pcDNA3ORF2- plasmid and GMCSF+ plasmid;

Cells transformed by baculoviruses not containing any nucleic acid insert coding for a PWD circovirus protein;

15 AIF SEPPIC adjuvant.

Batch C (F3):

- first injection

pcDNA3ORF1+ plasmid, pcDNA3ORF2+ plasmid and GMCSF+ plasmid;

20 - second and third injection (simultaneous)

pcDNA3ORF1+ plasmid, pcDNA3ORF2+ plasmid and GMCSF+ plasmid;

Cells transformed by BAC ORF1+ and BAC ORF2+ recombinant baculoviruses capable of respectively  
25 expressing the Rep protein of sequence SEQ ID No. 14 and the protein of sequence SEQ ID No. 15 of the PWD circovirus of TYPE B.

Batch D (F4) (control batch): no injection

30 The batches of piglets B, C and D are infected (tested) at the age of 6 weeks although batch A is not subjected to the test.

3) Observation of the batches

- counting of coughing/sneezing: 15 minutes/batch/day;

- consistency of fecal matter: every day;

35 - regular recordings: weekly taking of blood, weighing;

- weighing of food refuse: 3 times per week;

- calculation of the daily mean gain in weight (dmg);

The daily mean gains were calculated for each of the batches over a period of 28 days following testing (cf. Figure 10), an intermediate calculation of the dm<sub>g</sub> was likewise carried out for each of the  
 5 batches over the first and second periods of 14 days. The results obtained are reported below in Table 6.

Table 6: Daily mean gains

	F1	F2	F3	F4
d0-d14	411 g	450 g	511 g	461 g
d14-d28	623 g	362 g	601 g	443 g
d0-d28	554 g	406 g	556 g	452 g

10

- Measurement of hyperthermia

The measurement of hyperthermia, of greater than 41°C (cf. Figure 11) and greater than 40.2°C, was carried out for each of the batches over a total period  
 15 of 28 days following testing. The results obtained, corresponding to the ratio expressed as a percentage between the number of recordings of heat of greater than 41°C (or greater than 40.2°C) and the total number  
 20 of recordings of heat carried out on all of the pigs per one-week period are reported below in Tables 7 and 8, respectively for the hyperthermia measurements of greater than 41°C and greater than 40.2°C.

Table 7: Hyperthermia > 41°C

25

	F1	F2	F3	F4
W1	4.1	0.	0.	0.
W2	10.7	16.	0.	8.9
W3	4.7	27.	0.	45.
W4	0.	0.	0.	7.5

Table 8: Hyperthermia > 40.2

	F1	F2	F3	F4
W1	29.1	10.41	29.1	20.8
W2	28.5	39.2	10.7	37.5
W3	14.3	68.7	25.0	81.2
W4	3.3	17.5	20.0	55

## 4) Conclusion

5           The recordings carried out clearly show that  
the animals which received the three injections of a  
vaccine composition comprising nucleic acid fragments  
of PWD circovirus according to the invention and/or  
capable of expressing recombinant proteins of PWD  
10    circovirus, in particular of type B, did not exhibit  
hyperthermia (cf. Figure 10). These animals  
additionally did not experience a decline in their  
growth, the dmgs being comparable to those of  
uninfected control animals (cf. Figure 9). They did not  
15   exhibit any particular clinical sign.

These results demonstrate the efficacious  
protection of the piglets against infection with a PWD  
circovirus of the invention, the primary agent  
responsible for PWD or FPW, provided by a vaccine  
20   composition prepared from a nucleic acid fragment of  
the nucleic sequence of PWD circovirus according to the  
invention, in particular of type B, and/or from  
recombinant proteins encoded by these nucleic acid  
fragments.

25           These results in particular show that the  
proteins encoded by the ORF1 and ORF2 of PWD circovirus  
according to the invention are immunogenic proteins  
inducing an efficacious protective response for the  
prevention of infection by a PWD circovirus.



EXAMPLE 6: Serological diagnosis of PWD circovirus by immunodetermination using recombinant proteins or synthetic peptides of PWD circovirus

5 A - Serological diagnosis with recombinant proteins

The identification and the sequencing of porcine PWD circovirus allow recombinant proteins of PWD circovirus to be produced by the techniques of genetic recombination well known to the person skilled  
10 in the art.

By these techniques, recombinant proteins encoded, in particular, by the ORF'2 of the PWD circovirus, type B, were expressed by transformed Sf9 insect cells and then isolated.

15 These recombinant proteins encoded by the ORF'2 are extracted, after culture of the transformed Sf9 cells, by thermal cell lysis by means of 3 cycles of freezing/thawing to  $-70^{\circ}\text{C}/+37^{\circ}\text{C}$ . Healthy Sf9 cells or nontransformed control Sf9 cells are also lyzed.

20 These two antigenic fractions originating from nontransformed control Sf9 cells and Sf9 cells expressing the ORF'2 are precipitated at  $4^{\circ}\text{C}$  by a 60% plus or minus 5% saturated ammonium sulfate solution. Determination of total proteins is carried out with the  
25 aid of the Biorad kit. 500 ng of control Sf9 proteins and of semipurified Sf9 proteins expressing the ORF'2, in solution in 0.05 M bicarbonate buffer pH 9.6, are passively adsorbed at the bottom of 3 different cupules of a Nunc Maxisorp microplate by incubation for one  
30 night at  $+4^{\circ}\text{C}$ .

The reactivity of pig sera with respect to each of these antigenic fractions is evaluated by an indirect ELISA reaction of which the experimental protocol is detailed below:

- 35 - Saturation step: 200  $\mu\text{l}$ /cupule of PBS1X/3% semi-skimmed milk, 1 h 30 incubation at  $37^{\circ}\text{C}$ .  
- Washing: 200  $\mu\text{l}$ /cupule of PBS1X/Tween 20: 0.05%, 3 rapid washes.

- Serum incubation step: 100 µl/cupule of serum diluted to 1/100 in PBS1X/semi-skimmed milk, 1%/Tween 20: 0.05%, 1 h incubation at 37°C.
- Washing: 200 µl/cupule of PBS1X/Tween 20: 0.05%,  
5 2 rapid washes followed by 2 washes of 5 min.
- Conjugate incubation step: 50 µl/cupule of rabbit anti-pig conjugate diluted to 1/1000 in PBS1X/semi-skimmed milk, 1%/Tween 20: 0.05%, 1 h incubation at 37°C.
- 10 - Washing: 200 µl/cupule of PBS1X/Tween 20: 0.05%, 2 rapid washes followed by 2 washes of 5 min.
- Visualization step: 100 µl/cupule of OPD substrate/citrate buffer/H<sub>2</sub>O<sub>2</sub>, 15 min incubation at 37°C.
- 15 - Stopping of reaction: 50 µl/cupule of 1 N H<sub>2</sub>SO<sub>4</sub>.
- Reading in a spectrophotometer at 490 nm.

#### Results

The results obtained are shown below in Table 9.

20

Table 9

Antigens	Reactivity of Pig Serum not inoculated with Circovirus	Reactivity of Pig Serum inoculated with Circovirus
Purified Sf9 control	0.076	0.088
Sf9 expressing purified ORF'2	0.071	1.035

The results are expressed in optical density  
25 measured in a spectrophotometer at 490 nm during analysis by ELISA of the reactivity of pig sera which are or are not inoculated with the type B PWD circovirus according to the protocol indicated above.

#### B - Serological Diagnosis by Synthetic Peptide

30 The epitopic mapping of the proteins encoded,

for example, by the nucleic sequences ORF1 and ORF2 of the two types of PWD circovirus (types A and B) additionally allowed immunogenic circoviral epitopes to be identified on the proteins encoded by the nucleic sequences ORF'1 and ORF'2 as well as the specific epitopes of the protein encoded by the nucleic sequence ORF'2 of the type B PWD circovirus. Four specific epitopes of the type B PWD circovirus and one epitope common to the two types of PWD circovirus situated on the protein encoded by the nucleic sequence ORF'2 were synthesized in peptide form. The equivalent peptides in the circovirus of type A were likewise synthesized. All these peptides were evaluated as diagnostic antigens within the context of carrying out a serological test.

#### 15 Results

The results obtained are shown in Table 10 below.





133	GVGSSAVILDDNFVT	189	RGVGSTVVILDANFV
134	SAVILDDNFVTKATA	190	STVVILDANFVTPST
135	LDDNFVTKATALTYD	191	ILDANFVTPSTNLAY
136	FVTKATALTYDPYVN	192	NFVTPSTNLAYDPYI
137	ATALTYDPYVNYSSR	193	PSTNLAYDPYINYSS
138	TYDPYVNYSSRHTIT	194	LAYDPYINYSSRHTI
139	YVNYSSRHTITQPF	195	PYINYSSRHTIRQPF
140	SSRHTITQPFYSYHSR	196	YSSRHTIRQPFYHS
141	TITQPFYSYHSRYFTP	197	HTIRQPFYHSRYFT
142	PFSYHSRYFTPYPVL	198	QPFYHSRYFTPYPKPE
143	HSRYFTPYPVLDFTI	199	YHSRYFTPYPKPELDQT
144	FTPYPVLDFTIDYFQ	200	YFTPYPKPELDQTIDWF
145	PVLDFTIDYFQPNK	201	KPELDQTIDWFQPNK
146	FTIDYFQPNKRNQL	202	DQTIDWFQPNKRNQ
147	YFQPNKRNQLWLRL	203	DWFQPNKRNQLWLH
148	NNKRNQLWLRLQTAG	204	PNNKRNQLWLHLNTH
149	NQLWLRLQTAGNVDH	205	RNQLWLHLNTHTNVE
150	LRLQTAGNVDHVGLG	206	WLHLNTHTNVEHTGL
151	TAGNVDHVGLGTAFE	207	NHTNVEHTGLGYAL
152	VDHVGLGTAFENSIY	208	NVEHTGLGYALQNAT
153	GLGTAFENSIYDQEY	209	TGLGYALQNATTAQN
154	AFENSIYDQEYNIRV	210	YALQNATTAQNYVVR
155	SIYDQEYNIRVTMYV	211	NATTAQNYVVRLTIY
156	QEYNIRVTMYVQFRE	212	AQNYVVRLTIYVQFR
157	IRVTMYVQFRENFK	213	VVRLTIYVQREFIL
158	MYVQFRENFKDPPL	214	TIYVQREFILKDPL
159	VQFRENFKDPPLNP	215	YVQREFILKDPLNE

These peptides were synthesized according to the "spot" method which consists in simultaneous synthesis of a large number of peptides on a cellulose solid support, each site of synthesis of a peptide constituting a spot (Synt:em, NIMES). This method involves orientation of the peptides on the plate, these being fixed covalently by the carboxy-terminal end. A spot represents approximately 50 nmol of peptide.

The reference of the spots and corresponding peptide sequences is given in Table 11.

These membranes were used for immunoreactivity tests with respect to serum of SPF pigs which were or were not infected experimentally with the type B PWD circoviral strain as well as with respect to sera of infected pigs from conventional farms (conventional farms 1 or 2). This study allowed specific immunoreactive peptides of the circovirus of type B corresponding to the spots No. 121, No. 132, No. 133

and No. 152 (respectively of amino acid sequences SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19 and SEQ ID No. 20) to be demonstrated. An illustration is shown in Figure 12 where the membranes are visualized with an infected pig serum coming from a conventional farm. Nonspecific immunoreactive peptides of type [lacuna] were likewise demonstrated, among which we shall keep the peptide No. 146 which is strongly immunogenic.

A comparison between the peptide sequences of circoviruses of type A and B (Figure 13) indicates a divergence ranging from 20 to 60% for the specific immunoreactive peptides of the type B, and a weaker divergence (13%) between the nonspecific peptides.



Bibliographic references

- Allan, G.M. et al., 1995, Vet. Microbiol., 44: 49-64.
- Barany, F., 1911, PNAS. USA, 88: 189-193.
- Boulton, L.H. et al., 1997, J. Gen. Virol., 78 (Pt 6),  
5 1265-1270.
- Buckholz, R.G., 1993, Yeast systems for the expression  
of heterologous gene products. Curr. Op. Biotechnology  
4: 538-542.
- Burg, J.L. et al., 1996, Mol. and Cell. Probes, 10:  
10 257-271.
- Chu, B.C.F. et al., 1986, NAR, 14: 5591-5603.
- Chu, P.W.G. et al., 1993, Virus Research, 27: 161-171.
- Clark, E.G., 1997, American Association of Swine  
Practitioners, 499-501.
- 15 Daft, B. et al., 1996, American Association of  
Veterinary Laboratory Diagnosticians, 32.
- Derse, D. et al., 1995, J. Virol., 69(3): 1907-1912.
- Duck, P. et al., 1990, Biotechniques, 9: 142-147.
- Dulac, G.C. et al., 1989, Can. J. Vet. Res., 53:  
20 431-433.
- Edwards, C.P., and Aruffo, A., 1993, Current  
applications of COS cell based transient expression  
systems. Curr. Op. Biotechnology 4: 558-563.
- Edwards, S. et al., 1994, Vet. Rec., 134: 680-681.
- 25 Erlich, H.A., 1989, In PCR Technology. Principles and  
Applications for DNA Amplification. New York: Stockton  
Press.
- Felgner, et al., 1987, Proc. Natl. Acad. Sci., 84:  
7413.
- 30 Fontes, E.P.B. et al., 1994, J. Biol. Chem., Vol. 269,  
No. 11: 8459-8465.
- Fraley et al., 1980, J. Biol. Chem., 255: 10431.
- Guateli, J.C. et al., 1990, PNAS. USA, 87: 1874-1878.
- Hackland, A.F. et al., 1994, Arch. Virol., 139: 1-22.
- 35 Hanson, S.F. et al., 1995, Virology, 211: 1-9.
- Harding, J.C., 1997, American Association of Swine  
Practitioners, 503.

- Harding, R.M. et al., 1993, Journal of General Virology, 74: 323-328.
- Harding, J.C. and Clark, E.G., 1997, Swine Health and Production, Vol. 5, No. 5: 201-203.
- 5 Heyraud-Nitschke, F. et al., 1995, Nucleic Acids Research, Vol. 23, No. 6.
- Horner, G.W., 1991, Surveillance 18(5): 23.
- Houben-Weyl, 1974, in Methode der Organischen Chemie, E. Wunsch Ed., Volume 15-I and 15-II, Thieme, 10 Stuttgart.
- Huygen, K. et al., 1996, Nature Medicine, 2(8): 893-898.
- Innis, M.A. et al., 1990, in PCR Protocols. A guide to Methods and Applications, San Diego, Academic Press.
- 15 Kaneda, et al., 1989, Science, 243: 375.
- Kievitis, T. et al., 1991, J. Virol. Methods, 35: 273-286.
- Kohler, G. et al., 1975, Nature, 256(5517): 495-497.
- Kwoh, D.Y. et al., 1989, PNAS. USA, 86: 1173-1177.
- 20 Ladany, S. et al., 1989, J. Clin. Microbiol. 27: 2778-2783.
- Lazarowitz, S.G. et al., 1989, The EMBO Journal, Vol. 8 No. 4: 1023-1032.
- Luckow, V.A., 1993, Baculovirus systems for the 25 expression of human gene products. Curr. Op. Biotechnology 4: 564-572.
- Mankertz, A. et al., 1997, J. Virol., 71: 2562-2566.
- Matthews, J.A. et al., 1988, Anal. Biochem., 169: 1-25.
- McNeilly, F. et al., 1996, Vet. Immunol. Immunopathol., 30 49: 295-306.
- Meehan, B.M. et al., 1997, J. Gen. Virol. 78: 221-227.
- Merrifield, R.D., 1966, J. Am. Chem. Soc., 88(21): 5051-5052.
- Midoux, 1993, Nucleic Acids Research, 21: 871-878.
- 35 Miele, E.A. et al., 1983, J. Mol. Biol., 171: 281-295.
- Murphy, F.A. et al., 1995, Sixth Report of the International Committee on Taxonomy of Viruses. Springer-Verlag Wien New York.

- Nayar, G.P. et al., 1997, Can. Vet. J. 38(6): 385-386.
- Olins, P.O., and Lee, S.C., 1993, Recent advances in heterologous gene expression in E.coli. Curr. Op. Biotechnology 4: 520-525.
- 5 Pagano et al., 1967, J. Virol., 1: 891.
- Rolfs, A. et al., 1991, In PCR Topics. Usage of Polymerase Chain reaction in Genetic and Infectious Disease. Berlin: Springer-Verlag.
- Sambrook, J. et al., 1989, In Molecular cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- 10 Sanchez-Pescador, R., 1988, J. Clin. Microbiol., 26(10): 1934-1938.
- Segev D., 1992, in "Non-radioactive Labeling and Detection of Biomolecules". Kessler C. Springer Verlag, Berlin, New-York: 197-205.
- 15 Shiver, J.W., 1995, in Vaccines 1995, eds Chanock, R.M. Brown, F. Ginsberg, H.S. & Norrby, E., pp. 95-98, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- 20 Tascon, R.E. et al., 1996, Nature Medicine, 2(8): 888-892.
- Tischer, I. et al., 1982, Nature, 295: 64-66.
- Tischer, I. et al., 1986, Arch. Virol., 91: 271-276.
- 25 Tischer, I. et al., 1988, Zentralbl Bakteriell Mikrobiol Hyg [A] 270: 280-287.
- Tischer, I. et al., 1995, Arch. Virol., 140: 737-743.
- Urdea, M.S., 1988, Nucleic Acids Research, II: 4937-4957.
- 30 Walker, G.T. et al., 1992, NAR 20: 1691-1696.
- Walker, G.T. et al., 1992, PNAS. USA, 89: 392-396.
- White, B.A. et al., 1997, Methods in Molecular Biology, 67, Humana Press, Towota.
- Zhao, T.M. et al., 1996, Proc. Natl. Acad. Sci., USA
- 35 93(13): 6653-6648.

CLAIMS

1. Nucleotide sequence of the genome of PWD circovirus selected from the sequences SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 9, SEQ ID No. 10 or one of their fragments.
2. Nucleotide sequence of PWD circovirus, characterized in that it is selected from:
- a) a nucleotide sequence of a specific fragment of a sequence according to Claim 1;
  - b) a nucleotide sequence homologous to a nucleotide sequence such as defined in a);
  - c) a nucleotide sequence complementary to a nucleotide sequence such as defined in a) or b), and a nucleotide sequence of their corresponding RNA;
  - d) a nucleotide sequence capable of hybridizing under stringent conditions with a sequence such as defined in a), b) or c);
  - e) a nucleotide sequence comprising a sequence such as defined in a), b), c) or d); and
  - f) a nucleotide sequence modified by a nucleotide sequence such as defined in a), b), c), d) or e).
3. Nucleotide sequence according to Claim 2, characterized in that it is selected from the sequences SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13 or one of their fragments.
4. Nucleotide sequence according to Claim 2, characterized in that it comprises a nucleotide sequence selected from:
- a) a nucleotide sequence according to Claim 3;
  - b) a nucleotide sequence of a specific fragment of a sequence such as defined in a);
  - c) a homologous nucleotide sequence having at least 80% identity with a nucleotide sequence such as defined in a) or b);

d) a complementary nucleotide sequence or sequence of RNA corresponding to a sequence such as defined in a), b) or c); and

5 e) a nucleotide sequence modified by a sequence such as defined in a), b), c) or d).

5. Nucleotide sequence according to one of Claims 2 to 4, characterized in that the specific fragment nucleotide sequence comprises a nucleotide sequence selected from the following sequences:

- 10 a) 5' TGTGGCGA 3';  
b) 5' AGTTTCCT 3';  
c) 5' TCATTTAGAGGGTCTTTCAG 3';  
d) 5' GTCAACCT 3';  
e) 5' GTGGTTGC 3';  
15 f) 5' AGCCCAGG 3';  
g) 5' TTGGCTGG 3';  
h) 5' TCTAGCTCTGGT 3';  
i) 5' ATCTCAGCTCGT 3';  
j) 5' TGTCCTCCTCTT 3';  
20 k) 5' TCTCTAGA 3';  
l) 5' TGTACCAA 3';  
m) 5' TCCGTCTT 3';

and their complementary sequences.

25 6. Polypeptide encoded by a nucleotide sequence according to one of Claims 1 to 5.

7. Polypeptide according to Claim 6, characterized in that its sequence is represented by a specific fragment of one of the six sequences of amino acids shown in Figure 2 or in Figure 8.

30 8. Polypeptide according to Claim 6 or 7, characterized in that it is selected from the polypeptides of sequences SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16 or one of their fragments.

35 9. Polypeptide characterized in that it comprises a polypeptide selected from:

- a) a specific fragment of at least 5 amino acids of a polypeptide according to one of Claims 6 to 8;

- b) a polypeptide homologous to a polypeptide such as defined in a);
- c) a specific biologically active fragment of a polypeptide such as defined in a) or b); and
- 5 d) a polypeptide modified by a polypeptide such as defined in a), b) or c).

10. Polypeptide according to Claim 9, characterized in that it comprises a polypeptide selected from the polypeptides of sequences SEQ ID No. 17, SEQ ID No. 18,  
10 SEQ ID No. 19 and SEQ ID No. 20.

11. Nucleotide sequence coding for a polypeptide according to Claims 7 to 10.

12. Nucleotide sequence utilizable as a primer or probe, characterized in that said sequence is selected  
15 from the nucleotide sequences according to one of Claims 1 to 5 and 11.

13. Nucleotide sequence according to Claim 12, characterized in that said sequence is one of the primer of the pairs of primers selected from the  
20 following pairs:

- a) 5' GTG TGC TCG ACA TTG GTG TG 3', and  
5' TGG AAT GTT AAC GAG CTG AG 3';
- b) 5' GTG TGC TCG ACA TTG GTG TG 3', and  
5' CTC GCA GCC ATC TTG GAA TG 3';
- 25 c) 5' CGC GCG TAA TAC GAC TCA CT 3', and  
5' GTG TGC TCG ACA TTG GTG TG 3';
- d) 5' CGC GCG TAA TAC GAC TCA CT 3', and  
5' CTC GCA GCC ATC TTG GAA TG 3';
- e) 5' CCT GTC TAC TGC TGT GAG TAC CTT GT 3', and  
30 5' GCA GTA GAC AGG TCA CTC CGT TGT CC 3'.

14. Nucleotide sequence according to Claim 12, characterized in that said sequence is a specific consensus sequence of porcine circovirus other than PWD circovirus and in that it is one of the primers of the  
35 following pair of primers:

- a) 5' GTG TGC TCG ACA TTG GTG TG 3', and  
5' TGG AAT GTT AAC TAC CTC AA 3'.

15. Nucleotide sequence according to Claim 12, characterized in that said sequence is a specific consensus sequence of porcine circovirus other than PWD circovirus of type B and in that it is one of the primers of the following pair of primers:
- 5 a) 5' GGC GGC GCC ATC TGT AAC GGT TT 3' and  
5' GAT GGC GCC GAA AGA CGG GTA TC 3'.
16. Nucleotide sequence according to one of Claims 12 to 15, characterized in that it is labeled by a radioactive compound or by a nonradioactive compound.
17. Nucleotide sequence according to one of Claims 12 to 16, characterized in that it is covalently or noncovalently immobilized on a support.
18. Nucleotide sequence according to one of Claims 12 to 17, for the detection and/or the amplification of nucleic sequences.
19. Cloning and/or expression vector, characterized in that it contains a nucleotide sequence according to one of Claims 1 to 5 and 11.
20. Vector characterized in that it comprises a nucleotide sequence according to one of Claims 1 to 5 and 11, and in that it additionally comprises a gene of interest.
21. Viral pseudoparticle or particle generated from a vector according to one of Claims 19 and 20.
22. Host cell, characterized in that it is transformed by a vector according to one of Claims 19 and 20, or a viral particle according to Claim 21.
23. Animal, comprising a cell transformed according to Claim 22.
24. Procedure for preparation of a recombinant polypeptide, characterized in that it employs a vector according to one of Claims 19 and 20, a cell transformed by said vector and/or an animal comprising said transformed cell.
25. Procedure for preparation of a synthetic polypeptide, characterized in that it uses an amino



acid sequence of a polypeptide according to one of Claims 6 to 10.

26. Recombinant or synthetic polypeptide obtained by a procedure according to Claim 24 or 25.

5 27. Hybrid polypeptide, characterized in that it contains at least the sequence of a polypeptide according to one of Claims 6 to 10 and 26, and a sequence of a polypeptide capable of inducing an immune response in man or animals.

10 28. Hybrid polypeptide according to Claim 27, characterized in that it contains at least the sequence of a polypeptide according to one of Claims 6 to 10 and 26, and a sequence of a polypeptide capable of inducing a humoral and/or cellular response in man or animals.

15 29. Nucleotide sequence coding for a hybrid polypeptide according to one of Claims 27 and 28.

30. Vector characterized in that it contains a nucleotide sequence according to Claim 29.

20 31. Hybrid polypeptide according to one of Claims 27 and 28, characterized in that it is a recombinant polypeptide obtained by the employment of a vector according to Claim 30.

25 32. Procedure for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, in a biological sample, characterized in that it comprises the following steps:

30 a) contacting of the biological sample with a polypeptide according to one of Claims 6 to 10 and 26;

b) demonstration of the antigen-antibody complex possibly formed.

35 33. Procedure according to Claim 32 for the detection and/or identification of PWD circovirus of type B in a biological sample, characterized in that it comprises the following steps:

- a) contacting of the biological sample with a polypeptide according to Claim 10;
- b) demonstration of the antigen-antibody complex possibly formed.

5 34. Kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:

- 10 a) a polypeptide according to one of Claims 6 to 10 and 26;
- b) if need be, the reagents for the formation of the medium favorable to the immunological reaction;
- c) if need be, the reagents allowing demonstration of  
15 the antigen-antibody complexes possibly formed between the polypeptide(s) of the invention and the antibodies;
- d) if need be, a biological reference sample (negative control) devoid of antibodies recognized  
20 by said polypeptide;
- e) if need be, a biological reference sample (positive control) containing a predetermined quantity of antibodies recognized by said polypeptide.

25 35. Mono- or polyclonal antibodies, their fragments, or chimeric antibodies, characterized in that they are capable of specifically recognizing a polypeptide according to one of Claims 6 to 10 and 26.

30 36. Antibody according to Claim 35, characterized in that it is a labeled antibody.

37. Procedure for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, in a  
35 biological sample, characterized in that it comprises the following steps:

- a) contacting of the biological sample with an antibody according to one of Claims 35 or 36;

- b) demonstration of the antigen-antibody complex formed.

38. Kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus  
5 other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:

- a) a polyclonal or monoclonal antibody according to one of Claims 35 or 36;
- 10 b) if need be, the reagents for the formation of the medium favorable to the immunological reaction;
- c) the reagents allowing the demonstration of the antigen-antibody complexes produced by the immunological reaction.

15 39. Procedure for detection and/or identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, in a biological sample, characterized in that it employs a nucleotide sequence  
20 according to one of Claims 12 to 18.

40. Procedure according to Claim 39, characterized in that it contains the following steps:

- a) if need be, isolation of the DNA from the biological sample to be analyzed;
- 25 b) specific amplification of the DNA of PWD circovirus with the aid of at least one primer according to one of Claims 12 to 18;
- c) demonstration of the amplification products.

41. Procedure according to Claim 39, characterized  
30 in that it comprises the following steps:

- a) contacting of a nucleotide probe according to one of Claims 12 to 18 with a biological sample, the DNA contained in the biological sample having, if need be, previously been made accessible to  
35 hybridization under conditions allowing the hybridization of the probe with the DNA of the sample;

b) demonstration of the hybrid possibly formed between the nucleotide probe and the DNA of the biological sample.

42. Procedure according to Claim 39, characterized in that it comprises the following steps:

- 5 a) contacting of a nucleotide probe immobilized on a support according to Claim 17 with a biological sample, the DNA of the sample having, if need be, previously been made accessible to hybridization
- 10 under conditions allowing the hybridization of the probe with the DNA of the sample;
- b) contacting of the hybrid formed between the nucleotide probe immobilized on a support and the DNA contained in the biological sample, if need be
- 15 after elimination of the DNA of the biological sample which has not hybridized with the probe, with a nucleotide probe labeled according to Claim 16;
- c) demonstration of the novel hybrid formed in step
- 20 b).

43. Procedure according to Claim 41 or 42, characterized in that, previously to step a), the DNA of the biological sample is amplified with the aid of at least one primer according to one of Claims 12 to

25 15.

44. Kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:

30

- a) a nucleotide probe according to one of Claims 12 to 18;
- b) if need be, the reagents necessary for the carrying out of a hybridization reaction;
- 35 c) if need be, at least one primer according to one of Claims 12 to 18, as well as the reagents necessary for an amplification reaction of the DNA.

45. Kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:

- a) a nucleotide probe, a so-called capture probe, according to Claim 17;
- b) an oligonucleotide probe, called a revealing probe, according to Claim 16;
- 10 c) if need be, at least one primer according to one of Claims 12 to 18, as well as the reagents necessary for an amplification reaction of the DNA.

46. Kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:

- a) at least one primer according to one of Claims 12 to 18;
- 20 b) if need be, the reagents necessary for carrying out a DNA amplification reaction;
- c) if need be, a component allowing the sequence of the amplified fragment to be verified, more particularly an oligonucleotide probe according to
- 25 one of Claims 12 to 18.

47. Procedure or kit or set according to one of Claims 32 to 34, or 37 to 46, for the diagnosis of an infection by a PWD circovirus, by a porcine circovirus other than a PWD circovirus or by a porcine circovirus other than the PWD circovirus of type B.

48. Use of a nucleotide sequence according to one of Claims 1 to 5 and 11, of a polypeptide according to one of Claims 6 to 10 and 26, of an antibody according to one of Claims 35 and 36, of a cell according to Claim 22, and/or of an animal transformed according to Claim 23, for the selection of organic or inorganic compounds capable of modulating, inducing or inhibiting

the expression of genes, and/or of modifying the cellular replication of PWD circovirus or capable of inducing or of inhibiting in pigs the pathologies linked to an infection by a PWD circovirus.

5 49. Compound selection method capable of binding to a polypeptide according to one of Claims 6 to 10 and 26, capable of binding to a nucleotide sequence according to one of Claims 1 to 5 and 11, or capable of recognizing an antibody according to Claim 35, and/or  
10 capable of modulating, inducing or inhibiting the expression of genes, and/or of modifying the cellular replication of PWD circovirus, or capable of inducing or inhibiting in pigs the pathologies linked to an infection by a PWD circovirus, characterized in that it  
15 comprises the following steps:

a) contacting of said compound with said polypeptide, said nucleotide sequence, or with a cell transformed according to Claim 22, and/or administration of said compound to an animal  
20 transformed according to Claim 23;

b) determination of the activity of said compound.

50. Compound capable of being selected by a method according to Claim 49.

51. Pharmaceutical composition comprising a  
25 compound selected from the following compounds:

a) a nucleotide sequence according to one of Claims 1 to 5, 11 and 29;

b) a polypeptide according to one of Claims 6 to 10, 26 to 28 and 31;

30 c) a vector or a viral particle according to one of Claims 19 to 21 and 30, or a cell according to Claim 22;

d) an antibody according to Claim 35; and

e) a compound according to Claim 50.

35 52. Compound according to Claim 51, in combination with a pharmaceutically acceptable vehicle and, if need be, one or more adjuvants of the appropriate immunity.

53. Vaccine composition, characterized in that it comprises a compound selected from the following compounds:

- 5 a) a nucleotide sequence according to one of Claims 1 to 5, 11 and 29;
- b) a polypeptide according to one of Claims 6 to 10, 26 to 28 and 31;
- c) a vector or a viral particle according to one of Claims 19 to 21 and 30; and
- 10 d) a cell according to Claim 22.

54. Vaccine composition according to Claim 53, characterized in that it comprises a mixture of at least two of said compounds and in that one of the two said compounds is related to the PWD circovirus of type  
15 A and the other is related to the PWD circovirus of type B.

55. Vaccine composition, characterized in that it comprises at least one of the following compounds:

- 20 - a nucleotide sequence SEQ ID No. 11, SEQ ID No. 12, or one of their fragments;
- a polypeptide of sequence SEQ ID No. 14, SEQ ID No. 15, or one of their fragments;
- a vector or a viral particle comprising a nucleotide sequence SEQ ID No. 11, SEQ ID No. 12, or  
25 one of their fragments;
- a transformed cell capable of expressing a polypeptide of sequence SEQ ID No. 14, SEQ ID No. 15, or one of their fragments; or
- a mixture of at least two of said compounds.

30 56. Vaccine composition according to Claim 54 or 55, characterized in that it comprises said mixture of at least two of said compounds as a combination product for simultaneous, separate or protracted use for the prevention or the treatment of infection by a PWD  
35 circovirus.

57. Vaccine composition according to Claim 55 or 56, characterized in that said mixture comprises the following compounds:



- a pcDNA3 plasmid containing a nucleic acid of sequence SEQ ID No. 11;
  - a pcDNA3 plasmid containing a nucleic acid of sequence SEQ ID No. 12;
  - 5       - a pcDNA3 plasmid containing a nucleic acid coding for the GM-CSF protein;
  - a recombinant baculovirus containing a nucleic acid of sequence SEQ ID No. 11;
  - a recombinant baculovirus containing a nucleic acid of sequence SEQ ID No. 12; and
  - 10       - if need be, an adjuvant of the appropriate immunity, especially the adjuvant AIF<sup>TM</sup>.
58.       Pharmaceutical composition according to one of Claims 51 to 57, for the prevention or the treatment of
- 15       an infection by a PWD circovirus.
59.       Pharmaceutical composition according to one of Claims 54 to 58 for the prevention or the treatment of an infection by the PWD circovirus of type B.
60.       Use of a composition according to one of Claims 51 to 59 for the preparation of a medicament
- 20       intended for the prevention or the treatment of infection by a PWD circovirus.
61.       Use of a composition according to one of Claims 54 to 57 for the preparation of a medicament
- 25       intended for the prevention or the treatment of infection by the PWD circovirus of type B.
62.       Vector according to one of Claims 19, 20 and 30, viral particle according to Claim 21, or cell according to Claim 22, for the treatment and/or the
- 30       prevention of a disease by gene therapy.
63.       Use of a vector according to Claims 19, 20 and 30, of a viral particle according to Claim 21, or of a cell according to Claim 22, for the preparation of a medicament intended for the treatment and/or the
- 35       prevention of a disease by gene therapy.

Table 5: Summary of the measurements carried out during experimental reproduction of PWD. (The values of the control animals are reported in brackets, the underlined values indicate a difference between infected animals and control animals)

Measurement	Test	2	3	4	5	6	7
Status of the pigs	SPF	SPF	SPF	SPF	SPF	Conventional	Conventional
Age	CNEVA	field	CNEVA	CNEVA	CNEVA	5 weeks	6-7 weeks
Number	9 weeks	6 weeks	5 weeks	5 weeks	5 weeks	8	8
Inoculation route	4 Intratracheal route	6 Intratracheal route	12 Intratracheal + intramuscular route	8 Intratracheal + intramuscular route	8 Intratracheal + intramuscular route	8 Intratracheal + intramuscular route	8 Intratracheal + intramuscular route
Inoculum titer per pig	ND*	ND*	$10^{4.53}$ TCID <sub>50</sub> per ml: 1 ml IM + 5 ml IT	$10^{4.53}$ TCID <sub>50</sub> per ml: 1 ml IM + 5 ml IT	$10^{4.53}$ TCID <sub>50</sub> per ml: 1 ml IM + 5 ml IT	$10^{4.53}$ TCID <sub>50</sub> per ml: 1 ml IM + 5 ml IT	$10^{4.53}$ TCID <sub>50</sub> per ml: 1 ml IM + 5 ml IT
Start of hyperthermia	10 days post-infection	9-13 days post-infection	12-13 days post-infection	9-14 days post-infection	8-12 days post-infection	12 days post-infection	12 days post-infection
% of pigs in hyperthermia**	100%	83%	92%	100%	75%	88%	88%
Number of days of hyperthermia per pig**	7	4.5	3.3	5.8	7.5	11.6	11.6

Test	2	3	4	5	6	7
Measurement	40.4 to 41.7°C	40.6 to 42.3°C	40.2 to 41.6°C	40.3 to 40.8°C	40.6 to 42°C	40.2 to 41.9°C
Maximum temperatures ***						
Hyperthermia****						
% per week						
W1	3.5 (3.5)	17 (36)	7 (5)	37 (17)	16 (17)	20 (28)
W2	42 (3.5)	7 (13)	13 (1)	21 (3)	52 (10)	37 (28)
W3	35 (3.5)	33 (10)	28 (7)	62 (2)	34 (12)	79 (17)
W4	21 (3.5)	28 (7)	5 (0)	6 (3)	25 (22)	55 (3)
DMG:						
W1	928 (1053)	417 (357)	564 (620)	650 (589)	401 (407)	509 (512)
W2	678 (1028)	428 (617)	503 (718)	612 (584)	294 (514)	410 (310)
W3	661 (1000)	771 (642)	381 (657)	520 (851)	375 (586)	435 (440)
W4	786 (1100)	550 (657)	764 (778)	641 (696)	473 (610)	451 (681)
Contact pigs	Yes to 100%	Yes to 75%	Not tested	Not tested	Not tested	Not tested
transmission						
% of pulmonary lesions	25	75	0	25	25	12
% of ganglionic lesions	17	33	67	25	50	12

\* ND: not determined,

\*\* hyperthermia when the temperature is greater than 40°C,

\*\*\* range of maximum temperatures recorded at the individual level,

\*\*\*\* the percentage corresponds to the number of temperature recordings greater than 40°C divided by the total number of temperature recordings in the week on all of the pigs.

Table 10: Results of the evaluation as a diagnostic antigen of synthetic peptides encoded by the nucleic sequences ORF2 and ORF'2 of PWD circovirus of type A and B.

Infected pig serum reactivity						
Circovirus B						
Peptide	Type	Position	AA sequence	SPF D0/D54	Conventional 1 D0/D42	Conventional 2 D0/D42
Epic specificity						
circovirus						
121	B	71-85	VMMRFNINDFLPPG	+/-, +++	+/-, +++	-, +++
177	B	70-84	NVNELRFNIGQFLPP	+/-, +	+/-, +/-	+/-, -
131	B	115-129	QGDRGVGSSAVILDD	+/-, +/-	++, ++	+/-, +
188	A	114-127	TSNQRGVGSTVVIL	+/-, -	-, +/-	+/-, +/-
133	B	119-134	GVGSSAVILDDNVFTK	-, ++	++, +++	+/-, ++
189	A	118-132	RGVGSTVVILDANFV	+/-, -	-, +/-	+/-, +/-
146	B	171-185	FTIDYFQPNKRNQL	-, +/-	-, ++	-, ++
202	A	170-184	DQTIDWFQPNKRNQ	+++ , +++	+/-, ++	+, ++
152	B	195-209	VDHVGLGTAFENSIY	-, ++	+++ , +++	+/-, +
208	A	194-208	NVEHTGLGYALQNAT	-, -	-, -	-, -
Circovirus A&B						
Circovirus B						

+/-, +, ++, +++. Increasing intensities of the reactivities observed in Spot peptides on a nitrocellulose membrane. The porcine sera tested are from animals experimentally infected with the circovirus of type B within the animal houses of the CNEVA. Samples are taken from the animals before inoculation on d0 and 42 days or 54 days after inoculation, on d42, d54.



**Title of the Invention**

**CIRCOVIRUS SEQUENCES ASSOCIATED WITH PIGLET  
WEIGHT LOSS DISEASE (PWD)**

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**5 Information on Related Applications**

The present application claims the priority benefit, under 35 U.S.C. § 119, of International Application No. PCT/FR98/02634, filed December 4, 1998.

**Background of the Invention**

10 The invention relates to the genomic sequence and nucleotide sequences coding for polypeptides of PWD circovirus, such as the structural and nonstructural polypeptides of said circovirus, as well as vectors including said sequences and cells or animals transformed by these vectors. The invention likewise relates to methods for detecting these nucleic acids or polypeptides and kits for diagnosing infection by  
15 the PWD circovirus. The invention is also directed to a method for selecting compounds capable of modulating the viral infection. The invention further comprises pharmaceutical compositions, including vaccines, for the prevention and/or the treatment of viral infections by PWD circovirus as well as the use of a vector according to the invention for the prevention and/or the treatment of diseases  
20 by gene therapy.

Piglet weight loss disease (PWD), alternatively called fatal piglet wasting (FPW) has been widely described in North America (Harding, J.C., 1997), and authors have reported the existence of a relationship between this pathology and the presence of porcine circovirus (Daft, B. et al., 1996; Clark, E.G., 1997; Harding, J.C., 1997; Harding, J.C. and Clark, E.G., 1997; Nayar, G.P. et al., 1997). A  
25 porcine circovirus has already been demonstrated in established lines of cell cultures derived from pigs and chronically infected (Tischer, I., 1986, 1988, 1995; Dulac, G.C., 1989; Edwards, S., 1994; Allan, G.M., 1995 and McNeilly, F., 1996). This virus, during experimental infection of piglets, does not prove pathogenic for pigs

(Tischer, I., 1986, Horner, G.W., 1991) and its nucleotide sequence has been determined and characterized (Tischer, I., 1982; Meehan, B.M. et al., 1997; Mankertz., A., 1997). The porcine circovirus, called PCV virus, is part of the circovirus genus of the circoviridae family (Murphy, F.A. et al., 1995) whose virion has a circular DNA of size between 1.7 and 2.3 kb, which DNA comprises three open reading frames (ORF1 to ORF3), coding for a replication protein REP involved in the initiation and termination phase of rolling circular replication (RCR) (Heyraud-Nitschke, F., et al., 1995; Harding, M.R. et al., 1993; Hanson, S.F. et al., 1995; Fontes, E.P.B. et al., 1994), coding for a capsid protein (Boulton, L.H. et al., 1997; Hackland, A.F. et al., 1994; Chu, P.W.G. et al., 1993) and coding for a nonstructural protein called a dissemination protein (Lazarowitz., S.G. et al., 1989).

The authors of the present invention have noticed that the clinical signs perceptible in pigs and linked to infection by the PWD circovirus are very distinctive. These manifestations in general appear in pigs of 8 to 12 weeks of age, weaned for 4 to 8 weeks. The first signs are hypotonia without it being possible to speak of prostration. Rapidly (48 hours), the flanks hollow, the line of the spine becomes apparent, and the pigs "blanch." These signs are in general accompanied by hyperthermia, anorexia and most often by respiratory signs (coughing, dyspnea, polypnea). Transitory diarrhea can likewise appear. The disease state phase lasts approximately one month at the end of which the rate of mortality varies from 5 to 20%. To these mortalities, it is expedient to add a variable proportion (5-10%) of cadaveric animals which are no longer able to present an economic future. It is to be noted that outside of this critical stage of the end of post-weaning, no anomaly appears on the farms. In particular, the reproductive function is totally maintained.

On the epidemiological level, the first signs of this pathology appeared at the start of 1995 in the east of the Côtes d'Armor region in France, and the farms affected are especially confined to this area of the region. In December 1996, the number of farms concerned could not be evaluated with precision because of the

absence of a specific laboratory diagnostic method or of an epidemiological surveillance system of the livestock. Based on the clinical facts as well as on results of postmortem examinations supplied by veterinarians, it is possible to estimate this number as several dozen (80-100). The contagiousness of the disease is weak to moderate. Cases are being reported outside the initial area and for the majority are following the transfer of animals coming from farms familiar with the problem. On the other hand, a characteristic of the condition is its strong remanence. Thus, farms which have been affected for a year are still affected in spite of the massive administration of therapeutics. Farms with clinical expression are drawn from various categories of specialization (breeders/fatteners, post-weaners/ fatteners) and different economic structures are concerned. In addition, the disorders appear even in farms where the rules of animal husbandry are respected.

Numerous postmortem examinations have been carried out either on farms or in the laboratory. The elements of the lesional table are disparate. The most constant macroscopic lesions are pneumonia which sometimes appears in patchy form as well as hypertrophy of the lymphatic ganglia. The other lesions above all affect the thoracic viscera including, especially, pericarditis and pleurisy. However, arthritis and gastric ulcers are also observed. The lesions revealed in the histological examination are essentially situated at the pulmonary level (interstitial pneumonia), ganglionic level (lymphoid depletion of the lymph nodes, giant cells) and renal level (glomerulonephritis, vasculitis). The infectious agents have been the subject of wide research. It has been possible to exclude the intervention of pestiviruses and Aujeszky's disease. The disorders appear in the seropositive PDRS (Porcine Dysgenic and Respiratory Syndrome, an infection linked to an arteriovirus) herds, but it has not been possible to establish the role of the latter in the genesis of the disorders (the majority of the farms in Brittany are PDRS seropositive).

The authors of the present invention, with the aim of identifying the etiological agent responsible for PWD, have carried out "contact" tests between piglets which are obviously "ill" and SPF pigs (specific pathogen-free) from



5 CNEVA (Centre National d'Etudes Vétérinaires et Alimentaires, France). These tests allow the development of signs comparable to those observed on the farm to be observed in protected animal houses. The discrete signs such as moderate hyperthermia, anorexia and intermittent diarrhea appeared after one week of contact. It must be noted that the PDRS virus only diffused subsequent to the clinical signs. In addition, inoculations of organ homogenates of sick animals to healthy pigs allowed signs related to those observed on the farms to be reproduced, although with a lower incidence, linked to the favorable conditions of upkeep of the animals in the experimental installations.

10 Thus, the authors of the present invention have been able to demonstrate that the pathological signs appear as a well-defined entity affecting the pig at a particular stage of its growth.

This pathology has never been described in France. However, sparse information, especially Canadian, relates to similar facts.

15 The disorders cannot be mastered with the existing therapeutics.

The data collected both on the farm and by experimentation have allowed the following points to be highlighted:

- PWD is transmissible but its contagiousness is not very high,
- its etiological origin is of infectious and probably viral nature,
- 20 - PWD has a persistent character in the affected farms.

Considerable economic consequences ensue for the farms.

Thus, there is currently a significant need for a specific and sensitive diagnostic, whose production is practical and rapid, allowing the early detection of the infection.

25 A reliable, sensitive and practical test which allows the distinction between strains of porcine circovirus (PCV) is thus strongly desirable.

On the other hand, a need for efficient and well-tolerated treatment of infections with PWD circovirus likewise remains desirable, no vaccine currently being available against PWD circovirus.

Concerning PWD circovirus, it will probably be necessary to understand the role of the immune defense in the physiology and the pathology of the disease to develop satisfactory vaccines.

5 Fuller information concerning the biology of these strains, their interactions with their hosts, the associated infectivity phenomena and those of escape from the immune defenses of the host especially, and finally their implication in the development of associated pathologies, will allow a better understanding of these mechanisms. Taking into account the facts which have been mentioned above and which show in particular the limitations of combatting infection by the PWD  
10 circovirus, it is thus essential today on the one hand to develop molecular tools, especially starting from a better genetic knowledge of the PWD circovirus, and likewise to perfect novel preventive and therapeutic treatments, novel methods of diagnosis and specific, efficacious and tolerated novel vaccine strategies. This is precisely the subject of the present invention.

## 15 **Summary of the Invention**

The present invention relates to vaccines comprising a nucleotide sequence of the genome of Porcine circovirus type B, or a homologue or fragment thereof, and an acceptable pharmaceutical or veterinary vehicle. In one embodiment of the invention, the nucleotide sequence is selected from SEQ ID No. 15, SEQ ID No. 19  
20 SEQ ID No. 23, or SEQ ID No. 25, or a homologue or fragment thereof. In another embodiment of the invention, the homologue has at least 80% sequence identity to SEQ ID No. 15, SEQ ID No. 19, SEQ ID No. 23 or SEQ ID No. 25. In yet another embodiment, the vaccines further comprising an adjuvant

25 The present invention also relates to vaccines comprising a polypeptide encoded by a nucleotide sequence of the genome of PCVB, or a homologue or fragment thereof, and an acceptable pharmaceutical or veterinary vehicle. In one embodiment, the homologue has at least 80% sequence identity to SEQ ID No. 15, SEQ ID No. 19, SEQ ID No. 23 or SEQ ID No. 25. In another embodiment of the

invention, the nucleotide sequence is selected from SEQ ID No. 23 or SEQ ID No. 25, or a homologue or fragment thereof. In still another embodiment, the polypeptide has the amino acid sequence of SEQ ID No. 24 or SEQ ID No. 26. In yet another embodiment, the homologue has at least 80% sequence identity to SEQ ID No. 24 or SEQ ID No. 26. In another embodiment, the polypeptide has the amino acid sequence of SEQ ID No. 29, SEQ ID No. 30, SEQ ID No. 31, or SEQ ID No. 32.

5 A further aspect of the invention relates to vaccines comprising a vector and an acceptable pharmaceutical or veterinary vehicle, the vector comprising a nucleotide sequence of the genome of Porcine circovirus type B, or a homologue or fragment thereof. In one embodiment, the vaccine further comprises a gene coding for an expression product capable of inhibiting or retarding the establishment or development of a genetic or acquired disease.

10 The present invention also relates to vaccines comprising a cell and an acceptable pharmaceutical or veterinary vehicle, wherein the cell is transformed with a nucleotide sequence of the genome of Porcine circovirus type B, or a homologue or fragment thereof.

15 Still further, the present invention relates to vaccines comprising a pharmaceutically acceptable vehicle and a single polypeptide, wherein the single polypeptide consists of SEQ ID No. 26.

20 Additionally, the present invention relates to methods of immunizing a mammal against piglet weight loss disease comprising administering to a mammal an effective amount of the vaccines described above.

25 These and other aspects of the invention will become apparent to the skilled artisan in view of the teachings contained herein.

### **Brief Description of the Drawings**

Figure 1: Experimental scheme which has made it possible to bring about the isolation and the identification of the circovirus associated with PWD of type A and B.

5           Test 1: experimental reproduction of the PWD by inoculation of pig organ homogenates from farms affected by PWD.

          Test 2: experimental reproduction of PWD.

          Test 3: experimental reproduction of PWD.

          Test 4: no experimental reproduction of PWD.

10

Figure 2: Organization of the genome of the circovirus associated with PWD of type A (PCVA)

- strand of (+) polarity (SEQ ID No. 1);
- strand of (-) polarity (SEQ ID No. 5, represented according to the orientation
- 15           3' → 5');
- sequences of amino acids of proteins encoded by the two DNA strands in the three possible reading frames SEQ ID NOS: 2-4 and 6-8 respectively.

20           Figure 3: Alignment of the nucleotide sequence SEQ ID No. 1 of the PWD circovirus of type A (PCVA) and of the MEEHAN SEQ ID No. 163 strain and MANKERTZ SEQ ID No. 164 strain circoviruses of the porcine cell lines.

25           Figure 4: Alignment of the sequence of amino acids SEQ ID No. 10 of a polypeptide encoded by the nucleotide sequence SEQ ID No. 9 (ORF1) of the PWD circovirus of type A (PCVA) and of corresponding nucleotide sequences of the MEEHAN SEQ ID No. 165 strain and MANKERTZ SEQ ID No. 166 strain circoviruses of the porcine cell lines.

Figure 5: Alignment of the sequence of amino acids SEQ ID No. 12 of a polypeptide encoded by the nucleotide sequence SEQ ID No. 11 (ORF2) of the PWD circovirus of type A (PCVA) and of corresponding nucleotide sequences of the MEEHAN SEQ ID No. 167 strain and MANKERTZ SEQ ID No. 168 strain circoviruses of the porcine cell lines.

Figure 6: Alignment of the sequence of amino acids SEQ ID No. 14 of a polypeptide encoded by the nucleotide sequence SEQ ID No. 13 (ORF3) of the PWD circovirus of type A (PCVA) and of corresponding nucleotide sequences of the MEEHAN SEQ ID No. 169 strain and MANKERTZ SEQ ID No. 170 strain circoviruses of the porcine cell lines.

Figure 7: Western blot analysis of recombinant proteins of the PWD circovirus of type A (PCVA).

The analyses were carried out on cell extracts of Sf9 cells obtained after infection with recombinant baculovirus PCF ORF 1.

Figure 8: Organization of the genome of the circovirus associated with the PWD of type B (PCVB)

- strand of (+) polarity (SEQ ID No. 15);
- strand of (-) polarity (SEQ ID No. 19, represented according to the orientation 3' → 5');
- sequence of amino acids of proteins encoded by the two DNA strands in the three possible reading frames SEQ ID NOS: 16-18 and 20-22 respectively.

Figure 9: Evolution of the daily mean gain (DMG) of pig farms affected by piglet weight loss disease (PWD), placed under experimental conditions.

Figure 10: DMG compared for the 3 batches of pigs (F1, F3 and F4) calculated over a period of 28 days, after vaccination test.

5 Figure 11: Hyperthermia greater than 41°C, expressed as a percentage compared for the 3 batches of pigs (F1, F3 and F4) calculated per week over a period of 28 days, after vaccination test.

10 Figure 12: Membranes of peptide spots corresponding to the ORF2s revealed with the aid of an infected pig serum, originating from a conventional farm.

The numbers of specific peptides of the circovirus of type B as well as their nonreactive homologs (type A) are indicated in bold.

The nonspecific immunogenic peptides are indicated in italics.

15 Figure 13: Alignment of amino acid sequences of proteins encoded by the ORF2 of the PWD circovirus of type A SEQ ID No. 12 and by the ORF'2 of the PWD circovirus of type B SEQ ID No. 26. The position of 4 peptides corresponding to specific epitopes of the PWD circovirus of type B is indicated on the corresponding sequence by a bold line, their homolog on the sequence of the  
20 PWD circovirus of type A is likewise indicated by an ordinary line.

Figure 14: Charts the results of experiments that demonstrate, in terms of percent hyperthermia, that vaccination with ORF'1 and ORF'2 of PCV-B enhances the level of protection in swine challenged with PCV-B.

25

Figure 15: Charts the results of experiments that demonstrate, in terms of animal growth, that vaccination with ORF'1 and ORF'2 of PCV-B enhances the level of protection in swine challenged with PCV-B.

Figure 16: Immunoperoxidase staining of PK15 cells at 24 h post-transfection with the pcDNA3/ORF'2 plasmid. Expression of PCVB ORF'2 was confirmed by IPMA following incubation in the presence of the swine anti-PCVB monospecific serum

5

### **Detailed Description of the Invention**

The present invention relates to nucleotide sequences of the genome of PWD circovirus selected from the sequences SEQ ID No. 1, SEQ ID No. 5, SEQ ID No. 15, SEQ ID No. 19 or one of their fragments.

10 The nucleotide sequences of sequences SEQ ID No. 1 and SEQ ID No. 5 correspond respectively to the genome sequence of the strand of (+) polarity and of the strand of (-) polarity of the PWD circovirus of type A (or PCVA), the sequence SEQ ID No. 5 being represented according to the orientation 5'→3'.

15 The nucleotide sequences of sequences SEQ ID No. 15 and SEQ ID No. 19 correspond respectively to the genome sequence of the strand of (+) polarity and of the strand of (-) polarity of the PWD circovirus of type B (or PCVB), the sequence SEQ ID No. 19 being represented according to the orientation 5'→3'.

The present invention likewise relates to nucleotide sequences, characterized in that they are selected from:

- 20 a) a nucleotide sequence of a specific fragment of the sequence SEQ ID No. 1, SEQ ID No. 5, SEQ ID No. 15, SEQ ID No. 19 or one of their fragments;
- b) a nucleotide sequence homologous to a nucleotide sequence such as defined in a);
- c) a nucleotide sequence complementary to a nucleotide sequence such as  
25 defined in a) or b), and a nucleotide sequence of their corresponding RNA;
- d) a nucleotide sequence capable of hybridizing under stringent conditions with a sequence such as defined in a), b) or c);
- e) a nucleotide sequence comprising a sequence such as defined in a), b), c) or d); and



- f) a nucleotide sequence modified by a nucleotide sequence such as defined in a), b), c), d) or e).

Nucleotide, polynucleotide or nucleic acid sequence will be understood according to the present invention as meaning both a double-stranded or single-stranded DNA in the monomeric and dimeric (so-called in tandem) forms and the transcription products of said DNAs.

It must be understood that the present invention does not relate to the genomic nucleotide sequences taken in their natural environment, that is to say in the natural state. It concerns sequences which it has been possible to isolate, purify or partially purify, starting from separation methods such as, for example, ion-exchange chromatography, by exclusion based on molecular size, or by affinity, or alternatively fractionation techniques based on solubility in different solvents, or starting from methods of genetic engineering such as amplification, cloning and subcloning, it being possible for the sequences of the invention to be carried by vectors.

The nucleotide sequences SEQ ID No. 1 and SEQ ID No. 15 were obtained by sequencing of the genome by the Sanger method.

Nucleotide sequence fragment according to the invention will be understood as designating any nucleotide fragment of the PWD circovirus, type A or B, of length of at least 8 nucleotides, preferably at least 12 nucleotides, and even more preferentially at least 20 consecutive nucleotides of the sequence from which it originates.

Specific fragment of a nucleotide sequence according to the invention will be understood as designating any nucleotide fragment of the PWD circovirus, type A or B, having, after alignment and comparison with the corresponding fragments of known porcine circoviruses, at least one nucleotide or base of different nature. For example, the specific nucleotide fragments of the PWD circovirus of type A can easily be determined by referring to Figure 3 of the present invention in which the nucleotides or bases of the sequence SEQ ID No. 1 (circopordfp) are shown which

are of different nature, after alignment of said sequence SEQ ID No. 1 with the other two sequences of known porcine circovirus (circopormeeh and circopormank).

Homologous nucleotide sequence in the sense of the present invention is understood as meaning a nucleotide sequence having at least a percentage identity with the bases of a nucleotide sequence according to the invention of at least 80%, preferably 90% or 95%, this percentage being purely statistical and it being possible to distribute the differences between the two nucleotide sequences at random and over the whole of their length.

Specific homologous nucleotide sequence in the sense of the present invention is understood as meaning a homologous nucleotide sequence having at least one nucleotide sequence of a specific fragment, such as defined above. Said "specific" homologous sequences can comprise, for example, the sequences corresponding to the genomic sequence or to the sequences of its fragments representative of variants of PWD circovirus of type A or B. These specific homologous sequences can thus correspond to variations linked to mutations within strains of PWD circovirus of type A and B, and especially correspond to truncations, substitutions, deletions and/or additions of at least one nucleotide. Said homologous sequences can likewise correspond to variations linked to the degeneracy of the genetic code.

The term "degree or percentage of sequence homology" refers to "degree or percentage of sequence identity between two sequences after optimal alignment" as defined in the present application.

Two amino-acids or nucleotidic sequences are said to be "identical" if the sequence of amino-acids or nucleotidic residues, in the two sequences is the same when aligned for maximum correspondence as described below. Sequence comparisons between two (or more) peptides or polynucleotides are typically performed by comparing sequences of two optimally aligned sequences over a segment or "comparison window" to identify and compare local regions of sequence similarity. Optimal alignment of sequences for comparison may be conducted by the

local homology algorithm of Smith and Waterman, *Ad. App. Math* 2: 482 (1981),  
by the homology alignment algorithm of Neddleman and Wunsch, *J. Mol. Biol.* 48:  
443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl.*  
*Acad. Sci. (U.S.A.)* 85: 2444 (1988), by computerized implementation of these  
5 algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics  
Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison,  
WI), or by visual inspection.

"Percentage of sequence identity" (or degree or identity) is determined by  
comparing two optimally aligned sequences over a comparison window, where the  
10 portion of the peptide or polynucleotide sequence in the comparison window may  
comprise additions or deletions (i.e., gaps) as compared to the reference sequence  
(which does not comprise additions or deletions) **for optimal alignment** of the two  
sequences. The percentage is calculated by determining the number of positions at  
which the identical amino-acid residue or nucleic acid base occurs in both sequences  
15 to yield the number of matched positions, dividing the number of matched positions  
by the total number of positions in the window of comparison and multiplying the  
result by 100 to yield the percentage of sequence identity.

The definition of sequence identity given above is the definition that would  
use one of skill in the art. The definition by itself does not need the help of any  
20 algorithm, said algorithms being helpful only to achieve the optimal alignments of  
sequences, rather than the calculation of sequence identity.

From the definition given above, it follows that there is a well defined and  
only one value for the sequence identity between two compared sequences which  
value corresponds to the value obtained for the best or optimal alignment.

25 In the BLAST N or BLAST P "BLAST 2 sequence", software which is  
available in the web site <http://www.ncbi.nlm.nih.gov/gorf/bl2.html>, and habitually  
used by the inventors and in general by the skilled man for comparing and  
determining the identity between two sequences, gap cost which depends on the

sequence length to be compared is directly selected by the software (i.e. 11.2 for substitution matrix BLOSUM-62 for length  $> 85$ ).

5 In the present description, PWD circovirus will be understood as designating the circoviruses associated with piglet weight loss disease (PWD) of type A (PCVA) or type B (PCVB), defined below by their genomic sequence, as well as the circoviruses whose nucleic sequences are homologous to the sequences of PWD circoviruses of type A or B, such as in particular the circoviruses corresponding to variants of the type A or of the type B.

10 Complementary nucleotide sequence of a sequence of the invention is understood as meaning any DNA whose nucleotides are complementary to those of the sequence of the invention, and whose orientation is reversed (antiparallel sequence).

15 Hybridization under conditions of stringency with a nucleotide sequence according to the invention is understood as meaning a hybridization under conditions of temperature and ionic strength chosen in such a way that they allow the maintenance of the hybridization between two fragments of complementary DNA.

20 By way of illustration, conditions of great stringency of the hybridization step with the aim of defining the nucleotide fragments described above are advantageously the following.

The hybridization is carried out at a preferential temperature of 65°C in the presence of SSC buffer, 1 × SSC corresponding to 0.15 M NaCl and 0.05 M Na citrate. The washing steps, for example, can be the following:

25 - 2 × SSC, at ambient temperature followed by two washes with 2 × SSC, 0.5% SDS at 65°C; 2 × 0.5 × SSC, 0.5% SDS; at 65°C for 10 minutes each.

The conditions of intermediate stringency, using, for example, a temperature of 42°C in the presence of a 2 × SSC buffer, or of less stringency, for example a temperature of 37°C in the presence of a 2 × SSC buffer, respectively require a

globally less significant complementarity for the hybridization between the two sequences.

5 The stringent hybridization conditions described above for a polynucleotide with a size of approximately 350 bases will be adapted by the person skilled in the art for oligonucleotides of greater or smaller size, according to the teaching of Sambrook et al., 1989.

10 Among the nucleotide sequences according to the invention, those are likewise preferred which can be used as a primer or probe in methods allowing the homologous sequences according to the invention to be obtained, these methods, such as the polymerase chain reaction (PCR), nucleic acid cloning and sequencing, being well known to the person skilled in the art.

Among said nucleotide sequences according to the invention, those are again preferred which can be used as a primer or probe in methods allowing the presence of PWD circovirus or one of its variants such as defined below to be diagnosed.

15 The nucleotide sequences according to the invention capable of modulating, of inhibiting or of inducing the expression of PWD circovirus gene, and/or capable of modulating the replication cycle of PWD circovirus in the host cell and/or organism are likewise preferred. Replication cycle will be understood as designating the invasion and the multiplication of PWD circovirus, and its propagation from host cell to host cell in the host organism.

20 Among said nucleotide sequences according to the invention, those corresponding to open reading frames, called ORF sequences, and coding for polypeptides, such as, for example, the sequences SEQ ID No. 9 (ORF1), SEQ ID No. 11 (ORF2) and SEQ ID No. 13 (ORF3) respectively corresponding to the nucleotide sequences between the positions 47 and 985 determined with respect to the position of the nucleotides on the sequence SEQ ID No. 1, the positions 1723 and 1022 and the positions 658 and 38 with respect to the position of the nucleotides on the sequence SEQ ID No. 5 (represented according to the orientation 3'→5'), the ends being included, or alternatively the sequences SEQ ID No. 23 (ORF'1), SEQ

5 ID No. 25 (ORF'2) and SEQ ID No. 27 (ORF'3), respectively corresponding to the sequences between the positions 51 and 995 determined with respect to the position of the nucleotides on the sequence SEQ ID No. 15, the positions 1734 and 1033 and the positions 670 and 357, the positions being determined with respect to the position of the nucleotides on the sequence SEQ ID No. 19 (represented according to the orientation 3'→5'), the ends being included, are finally preferred.

10 The nucleotide sequence fragments according to the invention can be obtained, for example, by specific amplification, such as PCR, or after digestion with appropriate restriction enzymes of nucleotide sequences according to the invention, these methods in particular being described in the work of Sambrook et al., 1989. Said representative fragments can likewise be obtained by chemical synthesis when their size is not very large and according to methods well known to persons skilled in the art.

15 Modified nucleotide sequence will be understood as meaning any nucleotide sequence obtained by mutagenesis according to techniques well known to the person skilled in the art, and containing modifications with respect to the normal sequences according to the invention, for example mutations in the regulatory and/or promoter sequences of polypeptide expression, especially leading to a modification of the rate of expression of said polypeptide or to a modulation of the replicative cycle.

20 Modified nucleotide sequence will likewise be understood as meaning any nucleotide sequence coding for a modified polypeptide such as defined below.

25 The present invention relates to nucleotide sequences of PWD circovirus according to the invention, characterized in that they are selected from the sequences SEQ ID No. 9, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 23, SEQ ID No. 25, SEQ ID No. 27 or one of their fragments.

The invention likewise relates to nucleotide sequences characterized in that they comprise a nucleotide sequence selected from:

- a) a nucleotide sequence SEQ ID No. 9, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 23, SEQ ID No. 25, SEQ ID No. 27 or one of their fragments;



b) a nucleotide sequence of a specific fragment of a sequence such as defined in a);

c) a homologous nucleotide sequence having at least 80% identity with a sequence such as defined in a) or b);

5 d) a complementary nucleotide sequence or sequence of RNA corresponding to a sequence such as defined in a), b) or c); and

e) a nucleotide sequence modified by a sequence such as defined in a), b), c) or d).

10 As far as homology with the nucleotide sequences SEQ ID No. 9, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 23, SEQ ID No. 25, SEQ ID No. 27 or one of their fragments is concerned, the homologous, especially specific, sequences having a percentage identity with one of the sequences SEQ ID No. 9, SEQ ID No. 11, SEQ ID No. 13, SEQ ID No. 23, SEQ ID No. 25, SEQ ID No. 27 or one of their fragments of at least 80%, preferably 90% or 95%, are preferred. Said specific  
15 homologous sequences can comprise, for example, the sequences corresponding to the sequences ORF1, ORF2, ORF3, ORF'1, ORF'2 and ORF'3 of PWD circovirus variants of type A or of type B. In the same manner, these specific homologous sequences can correspond to variations linked to mutations within strains of PWD circovirus of type A or of type B and especially correspond to truncations,  
20 substitutions, deletions and/or additions of at least one nucleotide.

Among nucleotide sequences according to the invention, the sequence SEQ ID No. 23 which has a homology having more than 80% identity with the sequence SEQ ID No. 9, as well as the sequence SEQ ID No. 25, are especially preferred.

25 Preferably, the invention relates to the nucleotide sequences according to the invention, characterized in that they comprise a nucleotide sequence selected from the following sequences:

a) SEQ ID No. 33 170 5' TGTGGCGA 3';

b) SEQ ID No. 34 450 5' AGTTTCCT 3';

c) SEQ ID No. 35 1026 5' TCATTAGAGGGTCTTTCAG 3';



- d) SEQ ID No. 36    1074 5' GTCAACCT 3';  
e) SEQ ID No. 37    1101 5' GTGGTTGC 3';  
f) SEQ ID No. 38    1123 5' AGCCCCAGG 3';  
g) SEQ ID No. 39    1192 5' TTGGCTGG 3';  
5    h) SEQ ID No. 40    1218 5' TCTAGCTCTTGGT 3';  
i) SEQ ID No. 41    1501 5' ATCTCAGCTCGT 3';  
j) SEQ ID No. 42    1536 5' TGTCCTCCTCTT 3';  
k) SEQ ID No. 43    1563 5' TCTCTAGA 3';  
l) SEQ ID No. 44    1623 5' TGTACCAA 3';  
10    m) SEQ ID No. 45    1686 5' TCCGTCTT 3';

and their complementary sequences.

In the list of nucleotide sequences a)-m) above, the underlined nucleotides are mutated with respect to the two known sequences of circovirus which are nonpathogenic to pigs. The number preceding the nucleotide sequence represents the position of the first nucleotide of said sequence in the sequence SEQ ID No. 1.

The invention comprises the polypeptides encoded by a nucleotide sequence according to the invention, preferably a polypeptide whose sequence is represented by a fragment, especially a specific fragment, of one of the six sequences of amino acids represented in Figure 2, these six amino acid sequences corresponding to the polypeptides which can be encoded according to one of the three possible reading frames of the sequence SEQ ID No. 1 or of the sequence SEQ ID No. 5, or a polypeptide whose sequence is represented by a fragment, especially a specific fragment, of one of the six sequences of amino acids shown in Figure 8, these six sequences of amino acids corresponding to the polypeptides which can be encoded according to one of the three possible reading frames of the sequence SEQ ID No. 15 or of the sequence SEQ ID No. 19.

The invention likewise relates to the polypeptides, characterized in that they comprise a polypeptide selected from the amino acid sequences SEQ ID No. 10,

SEQ ID No. 12, SEQ ID No. 14, SEQ ID No. 24, SEQ ID No. 26, SEQ ID No. 28 or one of their fragments.

5 Among the polypeptides according to the invention, the polypeptide of amino acid sequence SEQ ID No. 24 which has a homology having more than 80% identity with the sequence SEQ ID No. 10, as well as the polypeptide of sequence SEQ ID No. 26, are especially preferred.

The invention also relates to the polypeptides, characterized in that they comprise a polypeptide selected from:

- 10 a) a specific fragment of at least 5 amino acids of a polypeptide of an amino acid sequence according to the invention;
- b) a polypeptide homologous to a polypeptide such as defined in a);
- c) a specific biologically active fragment of a polypeptide such as defined in a) or b); and
- 15 d) a polypeptide modified by a polypeptide such as defined in a), b) or c).

20 Among the polypeptides according to the invention, the polypeptides of amino acid sequences SEQ ID No. 29, SEQ ID No. 30, SEQ ID No. 31 and SEQ ID No. 32 are also preferred, these polypeptides being especially capable of specifically recognizing the antibodies produced during infection by the PWD circovirus of type B. These polypeptides thus have epitopes specific for the PWD circovirus of type B and can thus be used in particular in the diagnostic field or as immunogenic agent to confer protection in pigs against infection by PWD circovirus, especially of type B.

25 In the present description, the terms polypeptide, peptide and protein are interchangeable.

It must be understood that the invention does not relate to the polypeptides in natural form, that is to say that they are not taken in their natural environment but that they can be isolated or obtained by purification from natural sources, or else

obtained by genetic recombination, or alternatively by chemical synthesis and that they can thus contain unnatural amino acids, as will be described below.

Polypeptide fragment according to the invention is understood as designating a polypeptide containing at least 5 consecutive amino acids, preferably 10  
5 consecutive amino acids or 15 consecutive amino acids.

In the present invention, specific polypeptide fragment is understood as designating the consecutive polypeptide fragment encoded by a specific fragment nucleotide sequence according to the invention.

Homologous polypeptide will be understood as designating the polypeptides  
10 having, with respect to the natural polypeptide, certain modifications such as, in particular, a deletion, addition or substitution of at least one amino acid, a truncation, a prolongation, a chimeric fusion, and/or a mutation. Among the homologous polypeptides, those are preferred whose amino acid sequence has at least 80%, preferably 90%, homology with the sequences of amino acids of  
15 polypeptides according to the invention.

Specific homologous polypeptide will be understood as designating the homologous polypeptides such as defined above and having a specific fragment of polypeptide according to the invention.

In the case of a substitution, one or more consecutive or nonconsecutive  
20 amino acids are replaced by "equivalent" amino acids. The expression "equivalent" amino acid is directed here at designating any amino acid capable of being substituted by one of the amino acids of the base structure without, however, essentially modifying the biological activities of the corresponding peptides and such that they will be defined by the following.

25 These equivalent amino acids can be determined either by depending on their structural homology with the amino acids which they substitute, or on results of comparative tests of biological activity between the different polypeptides, which are capable of being carried out.

By way of example, the possibilities of substitutions capable of being carried out without resulting in an extensive modification of the biological activity of the corresponding modified polypeptides will be mentioned, the replacement, for example, of leucine by valine or isoleucine, of aspartic acid by glutamic acid, of  
5 glutamine by asparagine, of arginine by lysine etc., the reverse substitutions naturally being envisageable under the same conditions.

The specific homologous polypeptides likewise correspond to polypeptides encoded by the specific homologous nucleotide sequences such as defined above and thus comprise in the present definition the polypeptides which are mutated or  
10 correspond to variants which can exist in PWD circovirus, and which especially correspond to truncations, substitutions, deletions and/or additions of at least one amino acid residue.

Specific biologically active fragment of a polypeptide according to the invention will be understood in particular as designating a specific polypeptide  
15 fragment, such as defined above, having at least one of the characteristics of polypeptides according to the invention, especially in that it is:

- capable of inducing an immunogenic reaction directed against a PWD circovirus; and/or
- capable of being recognized by a specific antibody of a polypeptide  
20 according to the invention; and/or
- capable of linking to a polypeptide or to a nucleotide sequence of PWD circovirus; and/or
- capable of exerting a physiological activity, even partial, such as, for example, a dissemination or structural (capsid) activity; and/or
- 25 - capable of modulating, of inducing or of inhibiting the expression of PWD circovirus gene or one of its variants, and/or capable of modulating the replication cycle of PWD circovirus in the cell and/or the host organism.

The polypeptide fragments according to the invention can correspond to isolated or purified fragments naturally present in a PWD circovirus or correspond

to fragments which can be obtained by cleavage of said polypeptide by a proteolytic enzyme, such as trypsin or chymotrypsin or collagenase, or by a chemical reagent, such as cyanogen bromide (CNBr) or alternatively by placing said polypeptide in a very acidic environment, for example at pH 2.5. Such polypeptide fragments can likewise just as easily be prepared by chemical synthesis, from hosts transformed by an expression vector according to the invention containing a nucleic acid allowing the expression of said fragments, placed under the control of appropriate regulation and/or expression elements.

“Modified polypeptide” of a polypeptide according to the invention is understood as designating a polypeptide obtained by genetic recombination or by chemical synthesis as will be described below, having at least one modification with respect to the normal sequence. These modifications will especially be able to bear on amino acids at the origin of a specificity, of pathogenicity and/or of virulence, or at the origin of the structural conformation, and of the capacity of membrane insertion of the polypeptide according to the invention. It will thus be possible to create polypeptides of equivalent, increased or decreased activity, and of equivalent, narrower, or wider specificity. Among the modified polypeptides, it is necessary to mention the polypeptides in which up to 5 amino acids can be modified, truncated at the N- or C-terminal end, or even deleted or added.

As is indicated, the modifications of the polypeptide will especially have as objective:

- to render it capable of modulating, of inhibiting or of inducing the expression of PWD circovirus gene and/or capable of modulating the replication cycle of PWD circovirus in the cell and/or the host organism,
- of allowing its incorporation into vaccine compositions,
- of modifying its bioavailability as a compound for therapeutic use.

The methods allowing said modulations on eukaryotic or prokaryotic cells to be demonstrated are well known to the person skilled in the art. It is likewise well understood that it will be possible to use the nucleotide sequences coding for said

modified polypeptides for said modulations, for example through vectors according to the invention and described below, in order, for example, to prevent or to treat the pathologies linked to the infection.

5 The preceding modified polypeptides can be obtained by using combinatorial chemistry, in which it is possible to systematically vary parts of the polypeptide before testing them on models, cell cultures or microorganisms for example, to select the compounds which are most active or have the properties sought.

Chemical synthesis likewise has the advantage of being able to use:

- unnatural amino acids, or
- 10 - nonpeptide bonds.

Thus, in order to improve the duration of life of the polypeptides according to the invention, it may be of interest to use unnatural amino acids, for example in D form, or else amino acid analogs, especially sulfur-containing forms, for example.

15 Finally, it will be possible to integrate the structure of the polypeptides according to the invention, its specific or modified homologous forms, into chemical structures of polypeptide type or others. Thus, it may be of interest to provide at the N- and C-terminal ends compounds not recognized by the proteases.

20 The nucleotide sequences coding for a polypeptide according to the invention are likewise part of the invention.

The invention likewise relates to nucleotide sequences utilizable as a primer or probe, characterized in that said sequences are selected from the nucleotide sequences according to the invention.

25 Among the pairs of nucleotide sequences utilizable as a pair of primers according to the invention, the pairs of primers selected from the following pairs are preferred:

- a) SEQ ID No. 46 5' GTG TGC TCG ACA TTG GTG TG 3', and  
SEQ ID No. 47 5' TGG AAT GTT AAC GAG CTG AG 3';
- b) SEQ ID No. 46 5' GTG TGC TCG ACA TTG GTG TG 3', and

SEQ ID No. 48      5' CTC GCA GCC ATC TTG GAA TG 3';  
c) SEQ ID No. 49      5' CGC GCG TAA TAC GAC TCA CT 3', and  
SEQ ID No. 46      5' GTG TGC TCG ACA TTG GTG TG 3';  
d) SEQ ID No. 49      5' CGC GCG TAA TAC GAC TCA CT 3', and  
5      SEQ ID No. 48      5' CTC GCA GCC ATC TTG GAA TG 3'; and  
e) SEQ ID No. 50      5' CCT GTC TAC TGC TGT GAG TAC CTT GT 3',  
and  
SEQ ID No. 51      5' GCA GTA GAC AGG TCA CTC CGT TGT CC  
3'.

10      The cloning and the sequencing of the PWD circovirus, type A and B, has  
allowed it to be identified, after comparative analysis with the nucleotide sequences  
of other porcine circoviruses, that, among the sequences of fragments of these  
nucleic acids, were those which are strictly specific to the PWD circovirus of type  
A, of type B or of type A and B, and those which correspond to a consensus  
15      sequence of porcine circoviruses other than the PWD circoviruses of type A and/or  
B.

There is likewise a great need for nucleotide sequences utilizable as a primer  
or probe specific to the whole of the other known and nonpathogenic porcine  
circoviruses.

20      Said consensus nucleotide sequences specific to all circoviruses, other than  
PWD circovirus of type A and B, are easily identifiable from Figure 3 and the  
sequence SEQ ID No. 15, and are part of the invention.

Among said consensus nucleotide sequences, that which is characterized in  
that it is part of the following pair of primers is preferred:

25      a) SEQ ID No. 46      5' GTG TGC TCG ACA TTG GTG TG 3', and  
SEQ ID No. 52      5' TGG AAT GTT AAC TAC CTC AA 3'.

The invention likewise comprises a nucleotide sequence according to the  
invention, characterized in that said sequence is a specific consensus sequence of



porcine circovirus other than PWD circovirus of type B and in that it is one of the primers of the following pairs of primers:

- a) SEQ ID No. 53     5' GGC GGC GCC ATC TGT AAC GGT TT 3', and  
SEQ ID No. 54     5' GAT GGC GCC GAA AGA CGG GTA TC 3'.

5            It is well understood that the present invention likewise relates to specific polypeptides of known porcine circoviruses other than PWD circovirus, encoded by said consensus nucleotide sequences, capable of being obtained by purification from natural polypeptides, by genetic recombination or by chemical synthesis by procedures well known to the person skilled in the art and such as described in particular below. In the same manner, the labeled or unlabeled mono- or polyclonal antibodies directed against said specific polypeptides encoded by said consensus nucleotide sequences are also part of the invention.

15           It will be possible to use said consensus nucleotide sequences, said corresponding polypeptides as well as said antibodies directed against said polypeptides in procedures or sets for detection and/or identification such as described below, in place of or in addition to nucleotide sequences, polypeptides or antibodies according to the invention, specific to PWD circovirus type A and/or B.

20           These protocols have been improved for the differential detection of the circular monomeric forms of specific replicative forms of the virion or of the DNA in replication and the dimeric forms found in so-called in-tandem molecular constructs.

            The invention additionally relates to the use of a nucleotide sequence according to the invention as a primer or probe for the detection and/or the amplification of nucleic acid sequences.

25           The nucleotide sequences according to the invention can thus be used to amplify nucleotide sequences, especially by the PCR technique (polymerase chain reaction) (Erlich, 1989; Innis et al., 1990; Rolfs et al., 1991; and White et al., 1997).

These oligodeoxyribonucleotide or oligoribonucleotide primers advantageously have a length of at least 8 nucleotides, preferably of at least 12 nucleotides, and even more preferentially at least 20 nucleotides.

5 Other amplification techniques of the target nucleic acid can be advantageously employed as alternatives to PCR.

The nucleotide sequences of the invention, in particular the primers according to the invention, can likewise be employed in other procedures of amplification of a target nucleic acid, such as:

- 10 - the TAS technique (Transcription-based Amplification System), described by Kwoh et al. in 1989;
- the 3SR technique (Self-Sustained Sequence Replication), described by Guatelli et al. in 1990;
- the NASBA technique (Nucleic Acid Sequence Based Amplification), described by Kievitis et al. in 1991;
- 15 - the SDA technique (Strand Displacement Amplification) (Walker et al., 1992);
- the TMA technique (Transcription Mediated Amplification).

The polynucleotides of the invention can also be employed in techniques of amplification or of modification of the nucleic acid serving as a probe, such as:

- 20 - the LCR technique (Ligase Chain Reaction), described by Landegren et al. in 1988 and improved by Barany et al. in 1991, which employs a thermostable ligase;
- the RCR technique (Repair Chain Reaction), described by Segev in 1992;
- the CPR technique (Cycling Probe Reaction), described by Duck et al. in 25 1990;
- the amplification technique with Q-beta replicase, described by Miele et al. in 1983 and especially improved by Chu et al. in 1986, Lizardi et al. in 1988, then by Burg et al. as well as by Stone et al. in 1996.

In the case where the target polynucleotide to be detected is possibly an RNA, for example an mRNA, it will be possible to use, prior to the employment of an amplification reaction with the aid of at least one primer according to the invention or to the employment of a detection procedure with the aid of at least one probe of the invention, an enzyme of reverse transcriptase type in order to obtain a cDNA from the RNA contained in the biological sample. The cDNA obtained will thus serve as a target for the primer(s) or the probe(s) employed in the amplification or detection procedure according to the invention.

The detection probe will be chosen in such a manner that it hybridizes with the target sequence or the amplicon generated from the target sequence. By way of sequence, such a probe will advantageously have a sequence of at least 12 nucleotides, in particular of at least 20 nucleotides, and preferably of at least 100 nucleotides.

The invention also comprises the nucleotide sequences utilizable as a probe or primer according to the invention, characterized in that they are labeled with a radioactive compound or with a nonradioactive compound.

The unlabeled nucleotide sequences can be used directly as probes or primers, although the sequences are generally labeled with a radioactive element ( $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^3\text{H}$ ,  $^{125}\text{I}$ ) or with a nonradioactive molecule (biotin, acetylaminofluorene, digoxigenin, 5-bromodeoxyuridine, fluorescein) to obtain probes which are utilizable for numerous applications.

Examples of nonradioactive labeling of nucleotide sequences are described, for example, in French Patent No. 78.10975 or by Urdea et al. or by Sanchez-Pescador et al. in 1988.

In the latter case, it will also be possible to use one of the labeling methods described in patents FR-2 422 956 and FR-2 518 755.

The hybridization technique can be carried out in various manners (Matthews et al., 1988). The most general method consists in immobilizing the nucleic acid extract of cells on a support (such as nitrocellulose, nylon, polystyrene) and in

incubating, under well-defined conditions, the immobilized target nucleic acid with the probe. After hybridization, the excess of probe is eliminated and the hybrid molecules formed are detected by the appropriate method (measurement of the radioactivity, of the fluorescence or of the enzymatic activity linked to the probe).

5           The invention likewise comprises the nucleotide sequences according to the invention, characterized in that they are immobilized on a support, covalently or noncovalently.

10           According to another advantageous mode of employing nucleotide sequences according to the invention, the latter can be used immobilized on a support and can thus serve to capture, by specific hybridization, the target nucleic acid obtained from the biological sample to be tested. If necessary, the solid support is separated from the sample and the hybridization complex formed between said capture probe and the target nucleic acid is then detected with the aid of a second probe, a so-called detection probe, labeled with an easily detectable element.

15           Another subject of the present invention is a vector for the cloning and/or expression of a sequence, characterized in that it contains a nucleotide sequence according to the invention.

20           The vectors according to the invention, characterized in that they contain the elements allowing the expression and/or the secretion of said nucleotide sequences in a determined host cell, are likewise part of the invention.

25           The vector must then contain a promoter, signals of initiation and termination of translation, as well as appropriate regions of regulation of transcription. It must be able to be maintained stably in the host cell and can optionally have particular signals specifying the secretion of the translated protein. These different elements are chosen as a function of the host cell used. To this end, the nucleotide sequences according to the invention can be inserted into autonomous replication vectors within the chosen host, or integrated vectors of the chosen host.

          Such vectors will be prepared according to the methods currently used by the person skilled in the art, and it will be possible to introduce the clones resulting

therefrom into an appropriate host by standard methods, such as, for example, lipofection, electroporation and thermal shock.

The vectors according to the invention are, for example, vectors of plasmid or viral origin.

5 A preferred vector for the expression of polypeptides of the invention is baculovirus.

The vector pBS KS in which is inserted the in-tandem DNA sequence of the PWD circovirus type A (or DFP) as deposited at the CNCM on 3 July 1997, under the number I-1891, is likewise preferred.

10 These vectors are useful for transforming host cells in order to clone or to express the nucleotide sequences of the invention.

The invention likewise comprises the host cells transformed by a vector according to the invention.

15 These cells can be obtained by the introduction into host cells of a nucleotide sequence inserted into a vector such as defined above, then the culturing of said cells under conditions allowing the replication and/or expression of the transfected nucleotide sequence.

20 The host cell can be selected from prokaryotic or eukaryotic systems, such as, for example, bacterial cells (Olins and Lee, 1993), but likewise yeast cells (Buckholz, 1993), as well as animal cells, in particular the cultures of mammalian cells (Edwards and Aruffo, 1993), and especially Chinese hamster ovary (CHO) cells, but likewise the cells of insects in which it is possible to use procedures employing baculoviruses, for example (Luckow, 1993).

25 A preferred host cell for the expression of the proteins of the invention is constituted by sf9 insect cells.

A more preferred host cell according to the invention is E. coli, such as deposited at the CNCM on 3 July 1997, under the number I-1891.

The invention likewise relates to animals comprising one of said transformed cells according to the invention.

5 The obtainment of transgenic animals according to the invention overexpressing one or more of the genes of PWD circovirus or part of the genes will be preferably carried out in rats, mice or rabbits according to methods well known to the person skilled in the art, such as by viral or nonviral transfections. It will be possible to obtain the transgenic animals overexpressing one or more of said genes by transfection of multiple copies of said genes under the control of a strong promoter of ubiquitous nature, or selective for one type of tissue. It will likewise be possible to obtain the transgenic animals by homologous recombination in embryonic cell strains, transfer of these cell strains to embryos, selection of the affected chimeras at the level of the reproductive lines, and growth of said chimeras.

10 The transformed cells as well as the transgenic animals according to the invention are utilizable in procedures for preparation of recombinant polypeptides.

15 It is today possible to produce recombinant polypeptides in relatively large quantity by genetic engineering using the cells transformed by expression vectors according to the invention or using transgenic animals according to the invention.

20 The procedures for preparation of a polypeptide of the invention in recombinant form, characterized in that they employ a vector and/or a cell transformed by a vector according to the invention and/or a transgenic animal comprising one of said transformed cells according to the invention, are themselves comprised in the present invention.

25 Among said procedures for preparation of a polypeptide of the invention in recombinant form, the preparation procedures employing a vector, and/or a cell transformed by said vector and/or a transgenic animal comprising one of said transformed cells, containing a nucleotide sequence according to the invention coding for a polypeptide of PWD circovirus, are preferred.

The recombinant polypeptides obtained as indicated above can just as well be present in glycosylated form as in nonglycosylated form and can or cannot have the natural tertiary structure.



5 A preferred variant consists in producing a recombinant polypeptide used to a “carrier” protein (chimeric protein). The advantage of this system is that it allows a stabilization of and a decrease in the proteolysis of the recombinant product, an increase in the solubility in the course of renaturation in vitro and/or a simplification of the purification when the fusion partner has an affinity for a specific ligand.

More particularly, the invention relates to a procedure for preparation of a polypeptide of the invention comprising the following steps:

- a) culture of transformed cells under conditions allowing the expression of a recombinant polypeptide of nucleotide sequence according to the invention;
- 10 b) if need be, recovery of said recombinant polypeptide.

When the procedure for preparation of a polypeptide of the invention employs a transgenic animal according to the invention, the recombinant polypeptide is then extracted from said animal.

15 The invention also relates to a polypeptide which is capable of being obtained by a procedure of the invention such as described previously.

The invention also comprises a procedure for preparation of a synthetic polypeptide, characterized in that it uses a sequence of amino acids of polypeptides according to the invention.

20 The invention likewise relates to a synthetic polypeptide obtained by a procedure according to the invention.

The polypeptides according to the invention can likewise be prepared by techniques which are conventional in the field of the synthesis of peptides. This synthesis can be carried out in homogeneous solution or in solid phase.

25 For example, recourse can be made to the technique of synthesis in homogeneous solution described by Houben-Weyl in 1974.

This method of synthesis consists in successively condensing, two by two, the successive amino acids in the order required, or in condensing amino acids and fragments formed previously and already containing several amino acids in the appropriate order, or alternatively several fragments previously prepared in this



way, it being understood that it will be necessary to protect beforehand all the reactive functions carried by these amino acids or fragments, with the exception of amine functions of one and carboxyls of the other or vice-versa, which must normally be involved in the formation of peptide bonds, especially after activation of the carboxyl function, according to the methods well known in the synthesis of peptides.

According to another preferred technique of the invention, recourse will be made to the technique described by Merrifield.

To make a peptide chain according to the Merrifield procedure, recourse is made to a very porous polymeric resin, on which is immobilized the first C-terminal amino acid of the chain. This amino acid is immobilized on a resin through its carboxyl group and its amine function is protected. The amino acids which are going to form the peptide chain are thus immobilized, one after the other, on the amino group, which is deprotected beforehand each time, of the portion of the peptide chain already formed, and which is attached to the resin. When the whole of the desired peptide chain has been formed, the protective groups of the different amino acids forming the peptide chain are eliminated and the peptide is detached from the resin with the aid of an acid.

The invention additionally relates to hybrid polypeptides having at least one polypeptide according to the invention, and a sequence of a polypeptide capable of inducing an immune response in man or animals.

Advantageously, the antigenic determinant is such that it is capable of inducing a humoral and/or cellular response.

It will be possible for such a determinant to comprise a polypeptide according to the invention in glycosylated form used with a view to obtaining immunogenic compositions capable of inducing the synthesis of antibodies directed against multiple epitopes. Said polypeptides or their glycosylated fragments are likewise part of the invention.

These hybrid molecules can be formed, in part, of a polypeptide carrier molecule or of fragments thereof according to the invention, associated with a possibly immunogenic part, in particular an epitope of the diphtheria toxin, the tetanus toxin, a surface antigen of the hepatitis B virus (patent FR 79 21811), the  
 5 VP1 antigen of the poliomyelitis virus or any other viral or bacterial toxin or antigen.

The procedures for synthesis of hybrid molecules encompass the methods used in genetic engineering for constructing hybrid nucleotide sequences coding for the polypeptide sequences sought. It will be possible, for example, to refer  
 10 advantageously to the technique for obtainment of genes coding for fusion proteins described by Minton in 1984.

Said hybrid nucleotide sequences coding for a hybrid polypeptide as well as the hybrid polypeptides according to the invention characterized in that they are recombinant polypeptides obtained by the expression of said hybrid nucleotide  
 15 sequences are likewise part of the invention.

The invention likewise comprises the vectors characterized in that they contain one of said hybrid nucleotide sequences. The host cells transformed by said vectors, the transgenic animals comprising one of said transformed cells as well as the procedures for preparation of recombinant polypeptides using said vectors, said  
 20 transformed cells and/or said transgenic animals are, of course, likewise part of the invention.

The polypeptides according to the invention, the antibodies according to the invention described below and the nucleotide sequences according to the invention can advantageously be employed in procedures for the detection and/or  
 25 identification of PWD circovirus, or of porcine circovirus other than a PWD circovirus, in a biological sample (biological tissue or fluid) capable of containing them. These procedures, according to the specificity of the polypeptides, the antibodies and the nucleotide sequences according to the invention which will be used, will in particular be able to detect and/or to identify a PWD circovirus or a

porcine circovirus other than a PWD circovirus or other than the PWD circovirus of type B.

The polypeptides according to the invention can advantageously be employed in a procedure for the detection and/or the identification of PWD circovirus of type A, of type B, of type A or B, or porcine circovirus other than the PWD circovirus of type B, or of porcine circovirus other than the PWD circovirus of type A or B, in a biological sample (biological tissue or fluid) capable of containing them, characterized in that it comprises the following steps:

- a) contacting of this biological sample with a polypeptide or one of its fragments according to the invention (under conditions allowing an immunological reaction between said polypeptide and the antibodies possibly present in the biological sample);
- b) demonstration of the antigen-antibody complexes possibly formed.

In the present description, PWD circovirus, except if a particular mention is indicated, will be understood as designating a PWD circovirus of type A or of type B, and porcine circovirus other than PWD, except if a particular mention is indicated, will be understood as designating a porcine circovirus other than a PWD circovirus of type A and B.

Preferably, the biological sample is formed by a fluid, for example a pig serum, whole blood or biopsies.

Any conventional procedure can be employed for carrying out such a detection of the antigen-antibody complexes possibly formed.

By way of example, a preferred method brings into play immunoenzymatic processes according to the ELISA technique, by immunofluorescence, or radioimmunological processes (RIA) or their equivalent.

Thus, the invention likewise relates to the polypeptides according to the invention, labeled with the aid of an adequate label such as of the enzymatic, fluorescent or radioactive type.

Such methods comprise, for example, the following steps:

- deposition of determined quantities of a polypeptide composition according to the invention in the wells of a microtiter plate,
- introduction into said wells of increasing dilutions of serum, or of a biological sample other than that defined previously, having to be analyzed,
- 5       - incubation of the microplate,
- introduction into the wells of the microtiter plate of labeled antibodies directed against pig immunoglobulins, the labeling of these antibodies having been carried out with the aid of an enzyme selected from those which are capable of hydrolyzing a substrate by modifying the absorption of the radiation of the latter, at least at a determined wavelength, for example at 10       550 nm,
- detection, by comparison with a control test, of the quantity of hydrolyzed substrate.

15       The invention likewise relates to a kit or set for the detection and/or identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:

- a polypeptide according to the invention,
- if need be, the reagents for the formation of the medium favorable to the immunological or specific reaction,
- 20       - if need be, the reagents allowing the detection of the antigen-antibody complexes produced by the immunological reaction between the polypeptide(s) of the invention and the antibodies possibly present in the biological sample, these reagents likewise being able to carry a label, or to be recognized in their turn by a labeled reagent, more particularly in the case 25       where the polypeptide according to the invention is not labeled,
- if need be, a biological reference sample (negative control) devoid of antibodies recognized by a polypeptide according to the invention,

- if need be, a biological reference sample (positive control) containing a predetermined quantity of antibodies recognized by a polypeptide according to the invention.

5 The polypeptides according to the invention allow monoclonal or polyclonal antibodies to be prepared which are characterized in that they specifically recognize the polypeptides according to the invention. It will advantageously be possible to prepare the monoclonal antibodies from hybridomas according to the technique described by Kohler and Milstein in 1975. It will be possible to prepare the polyclonal antibodies, for example, by immunization of an animal, in particular a  
10 mouse, with a polypeptide or a DNA, according to the invention, associated with an adjuvant of the immune response, and then purification of the specific antibodies contained in the serum of the immunized animals on an affinity column on which the polypeptide which has served as an antigen has previously been immobilized. The polyclonal antibodies according to the invention can also be prepared by  
15 purification, on an affinity column on which a polypeptide according to the invention has previously been immobilized, of the antibodies contained in the serum of pigs infected by a PWD circovirus.

The invention likewise relates to mono- or polyclonal antibodies or their fragments, or chimeric antibodies, characterized in that they are capable of  
20 specifically recognizing a polypeptide according to the invention.

It will likewise be possible for the antibodies of the invention to be labeled in the same manner as described previously for the nucleic probes of the invention, such as a labeling of enzymatic, fluorescent or radioactive type.

The invention is additionally directed at a procedure for the detection and/or  
25 identification of PWD circovirus, of porcine circovirus other than a PWD circovirus, or other than the PWD circovirus of type B, in a biological sample, characterized in that it comprises the following steps:

- a) contacting of the biological sample (biological tissue or fluid) with a mono- or polyclonal antibody according to the invention (under conditions allowing

an immunological reaction between said antibodies and the polypeptides of PWD circovirus, of porcine circovirus other than a PWD circovirus, of porcine circovirus other than the PWD circovirus of type B, possibly present in the biological sample);

b) demonstration of the antigen-antibody complex possibly formed.

5 Likewise within the scope of the invention is a kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following components:

- 10 - a polyclonal or monoclonal antibody according to the invention, if need be labeled;
- if need be, a reagent for the formation of the medium favorable to the carrying out of the immunological reaction;
- if need be, a reagent allowing the detection of the antigen-antibody complexes produced by the immunological reaction, this reagent likewise being able to carry a label, or being capable of being recognized in its turn by a labeled reagent, more particularly in the case where said monoclonal or polyclonal antibody is not labeled;
- 15 - if need be, reagents for carrying out the lysis of cells of the sample tested.

20 The present invention likewise relates to a procedure for the detection and/or the identification of PWD, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, in a biological sample, characterized in that it employs a nucleotide sequence according to the invention.

25 More particularly, the invention relates to a procedure for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, in a biological sample, characterized in that it contains the following steps:

a) if need be, isolation of the DNA from the biological sample to be analyzed;



b) specific amplification of the DNA of the sample with the aid of at least one primer, or a pair of primers, according to the invention;

c) demonstration of the amplification products.

5 These can be detected, for example, by the technique of molecular hybridization utilizing a nucleic probe according to the invention. This probe will advantageously be labeled with a nonradioactive (cold probe) or radioactive element.

10 For the purposes of the present invention, "DNA of the biological sample" or "DNA contained in the biological sample" will be understood as meaning either the DNA present in the biological sample considered, or possibly the cDNA obtained after the action of an enzyme of reverse transcriptase type on the RNA present in said biological sample.

Another aim of the present invention consists in a procedure according to the invention, characterized in that it comprises the following steps:

15 a) contacting of a nucleotide probe according to the invention with a biological sample, the DNA contained in the biological sample having, if need be, previously been made accessible to hybridization under conditions allowing the hybridization of the probe with the DNA of the sample;

20 b) demonstration of the hybrid formed between the nucleotide probe and the DNA of the biological sample.

The present invention also relates to a procedure according to the invention, characterized in that it comprises the following steps:

25 a) contacting of a nucleotide probe immobilized on a support according to the invention with a biological sample, the DNA of the sample having, if need be, previously been made accessible to hybridization, under conditions allowing the hybridization of the probe with the DNA of the sample;

b) contacting of the hybrid formed between the nucleotide probe immobilized on a support and the DNA contained in the biological sample, if need



be after elimination of the DNA of the biological sample which has not hybridized with the probe, with a nucleotide probe labeled according to the invention;

c) demonstration of the novel hybrid formed in step b).

5 According to an advantageous embodiment of the procedure for detection and/or identification defined previously, this is characterized in that, prior to step a), the DNA of the biological sample is first amplified with the aid of at least one primer according to the invention.

10 The invention is additionally directed at a kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than the PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:

a) a nucleotide probe according to the invention;

b) if need be, the reagents necessary for the carrying out of a hybridization reaction;

15 c) if need be, at least one primer according to the invention as well as the reagents necessary for an amplification reaction of the DNA.

20 The invention likewise relates to a kit or set for the detection and/or the identification of PWD circovirus, of porcine circovirus other than a PWD circovirus or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following components:

a) a nucleotide probe, called a capture probe, according to the invention;

b) an oligonucleotide probe, called a revealing probe, according to the invention,

25 c) if need be, at least one primer according to the invention, as well as the reagents necessary for an amplification reaction of the DNA.

The invention also relates to a kit or set for the detection and/or identification of PWD circovirus, of porcine circovirus other than a PWD circovirus

or of porcine circovirus other than the PWD circovirus of type B, characterized in that it comprises the following elements:

- a) at least one primer according to the invention;
- b) if need be, the reagents necessary for carrying out a DNA  
5 amplification reaction;
- c) if need be, a component allowing the sequence of the amplified fragment to be verified, more particularly an oligonucleotide probe according to the invention.

10 The invention additionally relates to the use of a nucleotide sequence according to the invention, of a polypeptide according to the invention, of an antibody according to the invention, of a cell according to the invention, and/or of an animal transformed according to the invention, for the selection of an organic or inorganic compound capable of modulating, inducing or inhibiting the expression of genes, and/or of modifying the cellular replication of PWD circovirus or capable of  
15 inducing or of inhibiting the pathologies linked to an infection by a PWD circovirus.

The invention likewise comprises a method of selection of compounds capable of binding to a polypeptide or one of its fragments according to the invention, capable of binding to a nucleotide sequence according to the invention, or capable of recognizing an antibody according to the invention, and/or capable of  
20 modulating, inducing or inhibiting the expression of genes, and/or of modifying the cellular replication of PWD circovirus or capable of inducing or inhibiting the pathologies linked to an infection by a PWD circovirus, characterized in that it comprises the following steps:

- a) contacting of said compound with said polypeptide, said nucleotide  
25 sequence, or with a cell transformed according to the invention and/or administration of said compound to an animal transformed according to the invention;

- b) determination of the capacity of said compound to bind to said polypeptide or said nucleotide sequence, or to modulate, induce or inhibit the

expression of genes, or to modulate the growth or the replication of PWD circovirus, or to induce or inhibit in said transformed animal the pathologies linked to an infection by PWD circovirus (designated activity of said compound).

5 The compounds capable of being selected can be organic compounds such as polypeptides or carbohydrates or any other organic or inorganic compounds already known, or novel organic compounds elaborated by molecular modelling techniques and obtained by chemical or biochemical synthesis, these techniques being known to the person skilled in the art.

10 It will be possible to use said selected compounds to modulate the cellular replication of PWD circovirus and thus to control infection by this virus, the methods allowing said modulations to be determined being well known to the person skilled in the art.

15 This modulation can be carried out, for example, by an agent capable of binding to a protein and thus of inhibiting or of potentiating its biological activity, or capable of binding to an envelope protein of the external surface of said virus and of blocking the penetration of said virus into the host cell or of favoring the action of the immune system of the infected organism directed against said virus. This modulation can likewise be carried out by an agent capable of binding to a nucleotide sequence of a DNA of said virus and of blocking, for example, the expression of a polypeptide whose biological or structural activity is necessary for the replication or for the proliferation of said virus host cells to host cells in the host animal.

The invention relates to the compounds capable of being selected by a selection method according to the invention.

25 The invention likewise relates to a pharmaceutical composition comprising a compound selected from the following compounds:

- a) a nucleotide sequence according to the invention;
- b) a polypeptide according to the invention;

c) a vector, a viral particle or a cell transformed according to the invention;

d) an antibody according to the invention;

e) a compound capable of being selected by a selection method according to the invention;

possibly in combination with a pharmaceutically acceptable vehicle and, if need be, with one or more adjuvants of the appropriate immunity.

The invention also relates to an immunogenic and/or vaccine composition, characterized in that it comprises a compound selected from the following compounds:

a) a nucleotide sequence according to the invention;

b) a polypeptide according to the invention;

c) a vector or a viral particle according to the invention; and

d) a cell according to the invention.

In one embodiment, the vaccine composition according to the invention is characterized in that it comprises a mixture of at least two of said compounds a), b), c) and d) above and in that one of the two said compounds is related to the PWD circovirus of type A and the other is related to the PWD circovirus of type B.

In another embodiment of the invention, the vaccine composition is characterized in that it comprises at least one compound a), b), c), or d) above which is related to PWD circovirus of type B. In still another embodiment, the vaccine composition is characterized in that it comprises at least one compound a), b), c), or d) above which is related to PWD circovirus of type B ORF'2.

A compound related to the PWD circovirus of type A or of type B is understood here as respectively designating a compound obtained from the genomic sequence of the PWD circovirus of type A or of type B.

The invention is additionally aimed at an immunogenic and/or vaccine composition, characterized in that it comprises at least one of the following compounds:

- a nucleotide sequence SEQ ID No. 23, SEQ ID No. 25, or one of their fragments or homologues;
- a polypeptide of sequence SEQ ID No. 24, SEQ ID No. 26, or one of their fragments, or a modification thereof;
- 5       - a vector or a viral particle comprising a nucleotide sequence SEQ ID No. 23, SEQ ID No. 25, or one of their fragments or homologues;
- a transformed cell capable of expressing a polypeptide of sequence SEQ ID No. 24, SEQ ID No. 26, or one of their fragments, or a modification thereof; or
- 10       - a mixture of at least two of said compounds.

The invention also comprises an immunogenic and/or vaccine composition according to the invention, characterized in that it comprises said mixture of at least two of said compounds as a combination product for simultaneous, separate or protracted use for the prevention or the treatment of infection by a PWD circovirus, especially of type B.

15       In a preferred embodiment, the vaccine composition according to the invention comprises the mixture of the following compounds:

- a pcDNA3 plasmid containing a nucleic acid of sequence SEQ ID No. 23;
- a pcDNA3 plasmid containing a nucleic acid of sequence SEQ ID No. 25;
- 20       - a pcDNA3 plasmid containing a nucleic acid coding for the GM-CSF protein;
- a recombinant baculovirus containing a nucleic acid of sequence SEQ ID No. 23;
- a recombinant baculovirus containing a nucleic acid of sequence SEQ ID No. 25; and
- 25       - if need be, an adjuvant of the appropriate immunity, especially the adjuvant AIF<sup>TM</sup>.

The invention is likewise directed at a pharmaceutical composition according to the invention, for the prevention or the treatment of an infection by a PWD circovirus.

5 The invention is also directed at a pharmaceutical composition according to the invention for the prevention or the treatment of an infection by the PWD circovirus of type B.

10 The invention likewise concerns the use of a composition according to the invention, for the preparation of a medicament intended for the prevention or the treatment of infection by a PWD circovirus, preferably by the PWD circovirus of type B.

Under another aspect, the invention relates to a vector, a viral particle or a cell according to the invention, for the treatment and/or the prevention of a disease by gene therapy.

15 Finally, the invention comprises the use of a vector, of a viral particle or of a cell according to the invention for the preparation of a medicament intended for the treatment and/or the prevention of a disease by gene therapy.

20 The polypeptides of the invention entering into the immunogenic or vaccine compositions according to the invention can be selected by techniques known to the person skilled in the art such as, for example, depending on the capacity of said polypeptides to stimulate the T cells, which is translated, for example, by their proliferation or the secretion of interleukins, and which leads to the production of antibodies directed against said polypeptides.

25 In pigs, as in mice, in which a weight dose of the vaccine composition comparable to the dose used in man is administered, the antibody reaction is tested by taking of the serum followed by a study of the formation of a complex between the antibodies present in the serum and the antigen of the vaccine composition, according to the usual techniques.

The pharmaceutical compositions according to the invention will contain an effective quantity of the compounds of the invention, that is to say in sufficient

quantity of said compound(s) allowing the desired effect to be obtained, such as, for example, the modulation of the cellular replication of PWD circovirus. The person skilled in the art will know how to determine this quantity, as a function, for example, of the age and of the weight of the individual to be treated, of the state of advancement of the pathology, of the possible secondary effects and by means of a test of evaluation of the effects obtained on a population range, these tests being known in these fields of application.

According to the invention, said vaccine combinations will preferably be combined with a pharmaceutically acceptable vehicle and, if need be, with one or more adjuvants of the appropriate immunity.

Today, various types of vaccines are available for protecting animals or man against infectious diseases: attenuated living microorganisms (*M. bovis* - BCG for tuberculosis), inactivated microorganisms (influenza virus), acellular extracts (*Bordetella pertussis* for whooping cough), recombinant proteins (surface antigen of the hepatitis B virus), polysaccharides (pneumococcal). Vaccines prepared from synthetic peptides or genetically modified microorganisms expressing heterologous antigens are in the course of experimentation. More recently still, recombinant plasmid DNAs carrying genes coding for protective antigens have been proposed as an alternative vaccine strategy. This type of vaccination is carried out with a particular plasmid originating from a plasmid of *E.coli* which does not replicate *in vivo* and which codes uniquely for the vaccinating protein. Animals have been immunized by simply injecting the naked plasmid DNA into the muscle. This technique leads to the expression of the vaccine protein *in situ* and to an immune response of cellular type (CTL) and of humoral type (antibody). This double induction of the immune response is one of the principal advantages of the vaccination technique with naked DNA.

The vaccine compositions comprising nucleotide sequences or vectors into which are inserted said sequences are especially described in the international



application No. WO 90/11092 and likewise in the international application No. WO 95/11307.

5 The constitutive nucleotide sequence of the vaccine composition according to the invention can be injected into the host after having been coupled to compounds which favor the penetration of this polynucleotide into the interior of the cell or its transport to the cell nucleus. The resultant conjugates can be encapsulated in polymeric microparticles, as described in the international application No. WO 94/27238 (Medisorb Technologies International).

10 According to another embodiment of the vaccine composition according to the invention, the nucleotide sequence, preferably a DNA, is complexed with DEAE-dextran (Pagano et al., 1967) or with nuclear proteins (Kaneda et al., 1989), with lipids (Felgner et al., 1987) or encapsulated in liposomes (Fraley et al., 1980) or else introduced in the form of a gel facilitating its transfection into the cells (Midoux et al., 1993, Pastore et al., 1994). The polynucleotide or the vector  
15 according to the invention can also be in suspension in a buffer solution or be combined with liposomes.

Advantageously, such a vaccine will be prepared according to the technique described by Tacson et al. or Huygen et al. in 1996 or alternatively according to the technique described by Davis et al. in the international application No. WO  
20 95/11307.

Such a vaccine can likewise be prepared in the form of a composition containing a vector according to the invention, placed under the control of regulation elements allowing its expression in man or animal. It will be possible, for example, to use, by way of *in vivo* expression vector of the polypeptide antigen of  
25 interest, the plasmid pcDNA3 or the plasmid pcDNA1/neo, both marketed by Invitrogen (R&D Systems, Abingdon, United Kingdom). It is also possible to use the plasmid V1Jns.tPA, described by Shiver et al. in 1995. Such a vaccine will advantageously comprise, apart from the recombinant vector, a saline solution, for example a sodium chloride solution.

Pharmaceutically acceptable vehicle is understood as designating a compound or a combination of compounds entering into a pharmaceutical composition or vaccine which does not provoke secondary reactions and which allows, for example, the facilitation of the administration of the active compound, an increase in its duration of life and/or its efficacy in the body, an increase in its solubility in solution or alternatively an improvement in its conservation. These pharmaceutically acceptable vehicles are well known and will be adapted by the person skilled in the art as a function of the nature and of the mode of administration of the chosen active compound.

As far as the vaccine formulations are concerned, these can comprise adjuvants of the appropriate immunity which are known to the person skilled in the art, such as, for example, aluminum hydroxide, a representative of the family of muramyl peptides such as one of the peptide derivatives of N-acetyl muramyl, a bacterial lysate, or alternatively Freund's incomplete adjuvant.

These compounds can be administered by the systemic route, in particular by the intravenous route, by the intramuscular, intradermal or subcutaneous route, or by the oral route. In a more preferred manner, the vaccine composition comprising polypeptides according to the invention will be administered by the intramuscular route, through the food or by nebulization several times, staggered over time.

Their administration modes, dosages and optimum pharmaceutical forms can be determined according to the criteria generally taken into account in the establishment of a treatment adapted to an animal such as, for example, the age or the weight, the seriousness of its general condition, the tolerance to the treatment and the secondary effects noted. Preferably, the vaccine of the present invention is administered in an amount that is protective against piglet weight loss disease.

For example, in the case of a vaccine according to the present invention comprising a polypeptide encoded by a nucleotide sequence of the genome of PCV, or a homologue or fragment thereof, the polypeptide will be administered one time or several times, spread out over time, directly or by means of a transformed cell

capable of expressing the polypeptide, in an amount of about 0.1 to 10  $\mu\text{g}$  per kilogram weight of the animal, preferably about 0.2 to about 5  $\mu\text{g}/\text{kg}$ , more preferably about 0.5 to about 2  $\mu\text{g}/\text{kg}$  for a dose.

5 The present invention likewise relates to the use of nucleotide sequences of PWD circovirus according to the invention for the construction of autoreplicative retroviral vectors and the therapeutic applications of these, especially in the field of human gene therapy in vivo.

10 The feasibility of gene therapy applied to man no longer needs to be demonstrated and this relates to numerous therapeutic applications like genetic diseases, infectious diseases and cancers. Numerous documents of the prior art describe the means of employing gene therapy, especially through viral vectors. Generally speaking, the vectors are obtained by deletion of at least some of the viral genes which are replaced by the genes of therapeutic interest. Such vectors can be propagated in a complementation line which supplies in trans the deleted viral  
15 functions in order to generate a defective viral vector particle for replication but capable of infecting a host cell. To date, the retroviral vectors are amongst the most widely used and their mode of infection is widely described in the literature accessible to the person skilled in the art.

20 The principle of gene therapy is to deliver a functional gene, called a gene of interest, of which the RNA or the corresponding protein will produce the desired biochemical effect in the targeted cells or tissues. On the one hand, the insertion of genes allows the prolonged expression of complex and unstable molecules such as RNAs or proteins which can be extremely difficult or even impossible to obtain or to administer directly. On the other hand, the controlled insertion of the desired  
25 gene into the interior of targeted specific cells allows the expression product to be regulated in defined tissues. For this, it is necessary to be able to insert the desired therapeutic gene into the interior of chosen cells and thus to have available a method of insertion capable of specifically targeting the cells or the tissues chosen.

Among the methods of insertion of genes, such as, for example, microinjection, especially the injection of naked plasmid DNA (Derse, D. et al., 1995, and Zhao, T.M. et al., 1996), electroporation, homologous recombination, the use of viral particles, such as retroviruses, is widespread. However, applied in vivo, the gene transfer systems of recombinant retroviral type at the same time have a weak infectious power (insufficient concentration of viral particles) and a lack of specificity with regard to chosen target cells.

The production of cell-specific viral vectors, having a tissue-specific tropism, and whose gene of interest can be translated adequately by the target cells, is realizable, for example, by fusing a specific ligand of the target host cells to the N-terminal part of a surface protein of the envelope of PWD circovirus. It is possible to mention, for example, the construction of retroviral particles having the CD4 molecule on the surface of the envelope so as to target the human cells infected by the HIV virus (YOUNG, J.A.T. et al., Sciences 1990, 250, 1421-1423), viral particles having a peptide hormone fused to an envelope protein to specifically infect the cells expressing the corresponding receptor (KASAHARA, N. et al., Sciences 1994, 266, 1373-1376) or else alternatively viral particles having a fused polypeptide capable of immobilizing on the receptor of the epidermal growth factor (EGF) (COSSET, F.L. et al., J. of Virology 1995, 69, 10, 6314-6322). In another approach, single-chain fragments of antibodies directed against surface antigens of the target cells are inserted by fusion with the N-terminal part of the envelope protein (VALSESIA-WITTMAN, S. et al., J. of Virology 1996, 70, 3, 2059-2064; TEARINA CHU, T.H. et al., J. of Virology 1997, 71, 1, 720-725).

For the purposes of the present invention, a gene of interest in use in the invention can be obtained from a eukaryotic or prokaryotic organism or from a virus by any conventional technique. It is, preferably, capable of producing an expression product having a therapeutic effect and it can be a product homologous to the cell host or, alternatively, heterologous. In the scope of the present invention, a gene of interest can code for an (i) intracellular or (ii) membrane product present on the

surface of the host cell or (iii) secreted outside the host cell. It can therefore comprise appropriate additional elements such as, for example, a sequence coding for a secretion signal. These signals are known to the person skilled in the art.

5 In accordance with the aims pursued by the present invention, a gene of interest can code for a protein corresponding to all or part of a native protein as found in nature. It can likewise be a chimeric protein, for example arising from the fusion of polypeptides of various origins or from a mutant having improved and/or modified biological properties. Such a mutant can be obtained, by conventional biological techniques, by substitution, deletion and/or addition of one or more  
10 amino acid residues.

It is very particularly preferred to employ a gene of therapeutic interest coding for an expression product capable of inhibiting or retarding the establishment and/or the development of a genetic or acquired disease. A vector according to the invention is in particular intended for the prevention or for the treatment of cystic  
15 fibrosis, of hemophilia A or B, of Duchenne's or Becker's myopathy, of cancer, of AIDS and of other bacteria or infectious diseases due to a pathogenic organism: virus, bacteria, parasite or prion. The genes of interest utilizable in the present invention are those which code, for example, for the following proteins:

- a cytokine and especially an interleukin, an interferon, a tissue necrosis  
20 factor and a growth factor and especially a hematopoietic growth factor (G-CSF, GM-CSF),
- a factor or cofactor involved in clotting and especially factor VIII, von Willebrand's factor, antithrombin III, protein C, thrombin and hirudin,
- an enzyme or an enzyme inhibitor such as the inhibitors of viral proteases,
- 25 - an expression product of a suicide gene such as thymidine kinase of the HSV virus (herpesvirus) of type 1,
- an activator or an inhibitor of ion channels,
- a protein of which the absence, the modification or the deregulation of expression is responsible for a genetic disease, such as the CFTR protein,

dystrophin or minidystrophin, insulin, ADA (adenosine diaminose), glucocerebrosidase and phenylhydroxylase,

- a protein capable of inhibiting the initiation or the progression of cancers, such as the expression products of tumor suppressor genes, for example the P53 and Rb genes,
- a protein capable of stimulating an immune or an antibody response, and
- a protein capable of inhibiting a viral infection or its development, for example the antigenic epitopes of the virus in question or altered variants of viral proteins capable of entering into competition with the native viral proteins.

The invention thus relates to the vectors characterized in that they comprise a nucleotide sequence of PWD circovirus according to the invention, and in that they additionally comprise a gene of interest.

The present invention likewise relates to viral particles generated from said vector according to the invention. It additionally relates to methods for the preparation of viral particles according to the invention, characterized in that they employ a vector according to the invention, including viral pseudoparticles (VLP, virus-like particles).

The invention likewise relates to animal cells transfected by a vector according to the invention.

Likewise comprised in the invention are animal cells, especially mammalian, infected by a viral particle according to the invention.

The present invention likewise relates to a vector, a viral particle or a cell according to the invention, for the treatment and/or the prevention of a genetic disease or of an acquired disease such as cancer or an infectious disease. The invention is likewise directed at a pharmaceutical composition comprising, by way of therapeutic or prophylactic agent, a vector or a cell according to the invention, in combination with a vehicle acceptable from a pharmaceutical point of view.



Other characteristics and advantages of the invention appear in the examples and the figures.

The invention is described in more detail in the following illustrative examples. Although the examples may represent only selected embodiments of the invention, it should be understood that the following examples are illustrative and not limiting.

### Examples

EXAMPLE 1: Cloning, sequencing and characterization of the PWD circovirus of type A (PCVA)

#### 1. Experimental procedures

Experimental reproduction of the infection and its syndrome (cf. Figure 1).

A first test was carried out with pigs from a very well-kept farm, but affected by piglet weight loss disease (PWD), likewise called fatal piglet wasting (FPW). Tests carried out with SPF (specific pathogen-free) pigs showed a transfer of contaminant(s) finding expression in a complex pathology combining hyperthermia, retardation of growth, diarrhea and conjunctivitis. The PDRS (porcine dysgenic and respiratory syndrome) virus, an infectious disease due to an arteriovirus) was rapidly isolated from breeding pigs and contact pigs. It should have been possible to attribute all the clinical signs to the presence of the PDRS virus. However, two farm pigs presented signs of FPW without the PDRS virus being isolated. The histological analyses and blood formulas, however, showed that these pigs were suffering from an infectious process of viral origin.

In a second test, 8-week SPF pigs were inoculated by the intratracheal route with organ homogenates of two farm pigs suffering from FPW. The inoculated pigs exhibited hyperthermia 8 to 9 days post-infection, then their growth was retarded. Other SPF pigs, placed in contact, had similar, attenuated signs 30 days after the



initial experiment. No seroconversion with respect to a European or Canadian strain of PDRS virus was recorded in these animals.

A third test allowed the syndrome to be reproduced from samples taken from the pigs of the second test.

5            Conclusion

The syndrome is reproduced under the experimental conditions. It is determined by at least one infectious agent, which is transmittable by direct contact. The clinical constants are a sometimes high hyperthermia (greater than or equal to 41.5°C) which develops 8 to 10 days after infection. Retardation of the growth can be observed. The other signs are a reversal of the blood formula (reversal of the lymphocyte/polynuclear ratio from 70/30 to 30/70) and frequent lesions on the ganglia, especially those draining the respiratory apparatus (ganglionic hypertrophy, loss of structure with necrosis and infiltration by mononucleated or plurinucleated giant cells).

15          2. Laboratory studies

Various cell supports including primary pig kidney cells or cell lines, pig testicle cells, monkey kidney cells, pig lymphocytes, pig alveolar macrophages and circulating blood monocytes were used to demonstrate the possible presence of a virus. No cytopathic effect was demonstrated in these cells. On the other hand, the use of a serum of a pig sick after experimental infection allowed an intracellular antigen to be revealed in the monocytes, the macrophages and approximately 10% of pig kidney (PK) cells infected with organ homogenates. This indirect revealing was carried out kinetically at different culture times. It is evident from this that the antigen initially appears in the nucleus of the infected cells before spreading into the cytoplasm. The successive passages in cell culture did not allow the signal to be amplified.

Under electron microscopy on organ homogenates, spherical particles labeled specifically by the serum of sick pigs, infected under the experimental conditions, were visualized. The size of these particles is estimated at 20 nm.

After two passages of these organ homogenates over pig lymphocytes and then three passages over pig kidney or testicle cells, a cytopathic effect developed and was amplified. An adenovirus was visualized in the electron microscope, which, under the experimental conditions, did not reproduce FPW (only a hyperthermia peak was noted 24 to 48 hours after infection, and then nothing more).

It has been possible to demonstrate DNA bands in certain samples of pigs infected under the experimental conditions and having exhibited signs of the disease (results not shown). A certain connection exists between the samples giving a positive result in cell culture and those having a DNA band.

### Conclusion

At least two types of virus were demonstrated in the organ homogenates from pigs suffering from FPW. One is an adenovirus, but by itself alone it does not reproduce the disease. The other type of virus is a circovirus and is associated with FPW. This circovirus, of which two types have been isolated and sequenced, designated below PWD circovirus type A (or PCVA) and PWD circovirus of type B (or PCVB) have mutations with respect to the known sequences of circovirus which are nonpathogenic for the pig.

### 3. Cloning and sequencing of the DNA of the PWD circovirus of type A

Extraction of the replicative form (RF) DNA, cleavage by the Kpn I enzyme and amplification by a pair of primers flanking the Kpn I restriction site. Sequencing of the two strands at least twice by the Sanger method.

The nucleic sequence of the strand of (+) polarity of the genome of the PWD circovirus of type A (or PCVA), strain FPW, is represented by the sequence SEQ ID No. 1 in the list of sequences, the nucleic sequence of the strand of (-) polarity of the genome of the PWD circovirus of type A (or PCVA) being represented by the nucleic sequence 3' → 5' of Figure 3 or by the sequence SEQ ID No. 5 (represented according to the orientation 5' → 3') in the list of sequences.

The amino acid sequences SEQ ID No. 10, SEQ ID No. 12 and SEQ ID No. 14 of the list of sequences respectively represent the sequences of proteins

encoded by the nucleic sequences of the 3 open reading frames SEQ ID No. 9 (ORF1), corresponding to the REP protein, SEQ ID No. 11 (ORF2) and SEQ ID No. 13 (ORF3), determined from the sequence SEQ ID No. 1 of the strand of (+) polarity or of the nucleic sequence SEQ ID No. 5 of the strand of (-) polarity of the genome of the PWD circovirus of type A.

4. Comparison of the nucleotide sequences and amino acids of the PWD circovirus of type A (or associated with PWD) which are obtained with the corresponding sequences of MEEHAN and MANKERTZ circoviruses of porcine cell lines

10 Use of the DNA sequence analysis software, DNASIS.

Sequences of oligonucleotides used as primers or probes in the detection and/or identification procedures

1. Specific detection of the PWD circovirus of type A:

SEQ ID No. 46 primer PCV 5: 5' GTG TGC TCG ACA TTG GTG TG 3';

15 SEQ ID No. 47 primer PCV 10: 5' TGG AAT GTT AAC GAG CTG AG 3';

2. Specific detection of the circovirus of the cell lines:

SEQ ID No. 46 primer PCF 5: 5' GTG TGC TCG ACA TTG GTG TG 3';

SEQ ID No. 52 primer MEE 1: 5' TGG AAT GTT AAC TAC CTC AA 3';

3. Differential detection:

20 the pairs of primers used are those described, for example, in the paragraphs 1 and 2 above;

4. Detection of the monomeric circular replicative forms RF:

SEQ ID No. 46 primer PCV 5: 5' GTG TGC TCG ACA TTG GTG TG 3';

SEQ ID No. 48 primer PCV 6: 5' CTC GCA GCC ATC TTG GAA TG 3';

25 5. Detection of the vectors carrying the dimers in tandem:

Nar dimer:

SEQ ID No. 49 primer KS 620: 5' CGC GCG TAA TAC GAC TCA CT 3';

SEQ ID No. 46 primer PCV 5: 5' GTG TGC TCG ACA TTG GTG TG 3';

Kpn dimer:

30 SEQ ID No. 49 primer KS 620: 5' CGC GCG TAA TAC GAC TCA CT 3';

SEQ ID No. 48      primer PCV 6: 5'CTC GCA GCC ATC TTG GAA TG 3';

6. Differential detection:

The pairs of primers used are those described, for example, in paragraphs 4 and 5 above.

5      The procedures using the pairs or primers described in paragraphs 4 and 5 are of particular interest for differentially detecting the circular monomeric forms of specific replicative forms of the virion or of the DNA in replication and the dimeric forms found in the so-called in-tandem molecular constructs.

10      The in-tandem constructs of the viral genome (dimers) such as the constructs used for the preparation of the pBS KS + tandem PCV Kpn I vector, deposited at the CNM under the number I-1891, 3 July 1997 (E. coli transformed by said vector) are very interesting for their use in methods of production in sufficient quantity of an inoculum formed of DNA, intended for the virus production, this in the absence of a satisfactory virus production protocol in a cell system. These said  
15      methods of production using these in-tandem constructs of the viral genome will allow the virulence factors to be studied by mutation and by way of consequence will be able to be used for the production of a collection of viruses carrying the mutations indicated in the construction of vectors which will have the appropriate tropism and virulence. These vectors with autoreplicative structure have the sought  
20      gene transfer properties, especially for their applications in gene therapy, and in vaccinology.

Western-blot analysis of recombinant proteins of the PWD circovirus of type A

The results were obtained using a specific antiserum of the PWD circovirus produced during test 1 (cf. Figure 1).

25      Type of products analyzed.

The analyses were carried out on cell extracts of Sf9 cells obtained after infection by the recombinant baculovirus PCV ORF 1.

The culture of Sf9 cells was carried out in a 25 cm<sup>2</sup> Petri dish according to the standard culture methods for these cells. After centrifugation, the cell pellets are taken up with 300 µl of PBS buffer (phosphate saline buffer).

#### Electrophoresis (PAGE-SDS)

5 The electrophoresis is carried out on the cell extracts of Sf9 cells obtained previously on 5 samples (cf. Table 1 below) under the following conditions:

% polyacrylamide gel: 8%; conditions: denaturing

Voltage: 80 V; duration: 135 mn.

10 Table 1: Nature of the samples subjected to electrophoresis

Well No.	1	2	3	4	5
Sample applied	PM Rainbow	Raoul 24 h	Raoul 48 h	Raoul 72 h	Raoul 96 h
µl of sample	10	15	15	15	15
µl of Laemmli 4X	0	5	5	5	5

Legends to Table 1:

Laemmli 4X: loading buffer

PM Rainbow: molecular-weight markers (35, 52, 77, 107, 160 and 250 kD)

15 Raoul 24 h, 48 h, 72 h and 96 h: expression products of the ORF1 of the PWD circovirus of type A.

#### Western blot

20 After electrophoresis, the bands obtained in the different wells are transferred to nitrocellulose membrane for 1 h at 100 v in a TGM buffer (tris-glycine-methanol).

The Western blot is carried out under the following conditions:

1) Saturation with a solution containing 5% of skimmed milk; 0.05% of Tween 20 in a TBS 1X buffer (tris buffer saline) for 30 min.

25 2) 1st antibody:

10 ml of PWD anticircovirus antibody of type A are added diluted to 1/100, then the reaction mixture is incubated for one night at 4°C. Three washes of 10 min in TBS 1X are carried out.

3) 2nd antibody:

5 10 ml of pig rabbit P164 antibody anti-immunoglobulins, coupled to peroxidase (Dakopath) are added diluted to 1/100, then the reaction medium is incubated for 3 hours at 37°C. Three washes of 10 min in TBS 1X are carried out.

4) Visualization

10 The substrate 4-chloro-1-naphthol in the presence of oxygenated water is used for visualization.

Results

The results are shown in Figure 7.

15 Kinetics of appearance of antibodies specific for the REP recombinant protein of the PWD circovirus of type A expressed in baculovirus after infection of pigs by the PWD circovirus of type A (test 4, cf. Figure 1)

After infection of the pigs, a sample of serum of each of the infected pigs is taken at different periods expressed in the table by the date of taking (carried out here in the same year) and is then analyzed by Western blot.

The visualization of the specific antibodies is carried out in the manner described previously.

The results obtained are shown by Table 2 below.

Table 2: Kinetics of appearance of specific antibodies

Sample	Pigs	10/6	16/06	23/06	01/07	08/07	15/07	21/07
A3 Control	1						Neg.	
	2						Neg.	
B2 Infec. RP+	1	Neg.	Neg.	Neg.	+	+	++	+++
	2	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
	3	Neg.	Neg.	Neg.	Neg.	+	+	+
	4	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	++

Legends to Table 2:

A3 control: uninfected control animals;

B2 Infec. RP+: animals infected with pig kidney (PK) cells containing the circovirus;

5 Neg.: negative;

+, ++, +++: intensity scale of the positive reaction;

10/06, 16/06, 23/06, 01/07, 08/07, 15/07, 21/07: dates expressed in day/month on which the different withdrawals of serum were carried out.

10 EXAMPLE 2: Cloning, sequencing and characterization of the type B PWD circovirus (PCVB)

The techniques used for cloning, sequencing and characterization of the type B PWD circovirus (PCVB) are those used in Example 1 above for the type A PWD circovirus (PCVA).

15 The nucleic sequence of the strand of (+) polarity of the genome of the PWD circovirus of type B (or PCVB) is represented by the sequence SEQ ID No. 15 in the sequence listing, the nucleic sequence of the strand of (-) polarity of the genome of the PWD circovirus of type B (or PCVB) being represented by the nucleic sequence 3' → 5' of Figure 8 or by the sequence SEQ ID No. 19  
20 (represented according to the orientation 5' → 3') in the sequence listing.

The amino acid sequences SEQ ID No. 24, SEQ ID No. 26 and SEQ ID No. 28 of the sequence listing respectively represent the sequences of the proteins encoded by the nucleic sequences of the 3 open reading frames SEQ ID No. 23 (ORF'1), corresponding to the REP protein, SEQ ID No. 25 (ORF'2) and SEQ ID  
25 No. 27 (ORF'3), determined from the sequence SEQ ID No. 15 of the strand of (+) polarity or from the nucleic sequence SEQ ID No. 19 of the strand of (-) polarity of the genome of the PWD circovirus of type B.

30 EXAMPLE 3: Comparative analysis of nucleotide sequences (ORF1, ORF2 and genomic) and amino acid sequences encoded by the ORF1 and the ORF2 of the PWD circoviruses of type A (PCVA) and of type B (PCVB)



The results expressed in % of homology are shown in Tables 3 and 4 below.

Table 3: Compared analysis of the amino acid sequences

% homology	ORF1	ORF2
PCVA/PCVB	80.4	56.2

5

Table 4: Compared analysis of the nucleotide sequences

% homology	Genomic	ORF1	ORF2	The remainder
PCVA/PCVB	70.4	80.4	60.1	66.1

EXAMPLE 4: Observation of the disease and reproduction of the disease under experimental conditions

a) Test No. 1: Observation of the disease

10

The objective is to take breeding animals at the start of disease and to place them under experimental conditions to follow the progression of the pathology and describe all the clinical signs thereof. This first test was carried out on 3 breeding pigs aged 10 weeks of which 2 were already ill (suffering from wasting), and on 3 other pigs aged 13 weeks, not having signs of disease. The clinical observation was spread over a period of 37 days. Two pigs of 10 weeks wasted rapidly (pigs 1 and 2, Figure 9) and had to be painlessly killed 5 and 6 days after their arrival. A single pig exhibited hyperthermia over 5 days and diarrhea. Two other pigs exhibited dyspnea and cough, of which one additionally had hyperthermia, greater than 41°C, for the two first days of its stay. Another pig had retarded growth in the second week (pig 6, Figure 9), without any other clinical sign being recorded. On the

15

20

lesional level, 5 pigs out of 6 exhibited macroscopic lesions of gray pneumonia, the sixth exhibited cicatricial lesions on the lung.

b) Test No. 2: Reproduction of the disease from inocula prepared in farm pigs.

The two sick pigs in test 1 served to prepare inocula which were tested in test 2 on specific-pathogen-free (SPF) pigs. The SPF pigs were aged 9 weeks at the time of inoculation. The clinical and lesional results are shown in Table 5.

**Table 5:** Summary of the measurements carried out during experimental reproduction of PWD. (The values of the control animals are reported in brackets, the underlined values indicate a difference between infected animals and control animals)

Measurement	Test	2	3	4	5	6	7
Status of the pigs		SPF	SPF	SPF	SPF	Conventional	Conventional
Age		CNEVA	field	CNEVA	CNEVA	5 weeks	6-7 weeks
Number		9 weeks	6 weeks	5 weeks	5 weeks	8	8
Inoculation route		Intratracheal route	Intratracheal route	Intratracheal + intramuscular route	Intratracheal + intramuscular route	Intratracheal + intramuscular route	Intratracheal + intramuscular route
Inoculum titer per pig		ND*	ND*	$10^{4.53}$ TCID <sub>50</sub> per ml: 1 ml IM + 5 ml IT	$10^{4.53}$ TCID <sub>50</sub> per ml: 1 ml IM + 5 ml IT	$10^{4.53}$ TCID <sub>50</sub> per ml: 1 ml IM + 5 ml IT	$10^{4.53}$ TCID <sub>50</sub> per ml: 1 ml IM + 5 ml IT
Start of hyperthermia		10 days post-infection	9-13 days post-infection	12-13 days post-infection	9-14 days post-infection	8-12 days post-infection	12 days post-infection
% of pigs in hyperthermia**		100%	83%	92%	100%	75%	88%
Number of days of hyperthermia per pig**		7	4.5	3.3	5.8	7.5	11.6

Maximum temperatures | 40.4 to 41.7°C | 40.6 to 42.3°C | 40.2 to 41.6°C | 40.3 to 40.8°C | 40.6 to 42°C | 40.2 to 41.9°C |

Measurement	Test	2	3	4	5	6	7
*** Hyperthermia**** % per week							
W1		3.5 (3.5)	17 (36)	7 (5)	37 (17)	16 (17)	20 (28)
W2		<u>42 (3.5)</u>	7 (13)	<u>13 (1)</u>	<u>21 (3)</u>	<u>52 (10)</u>	37 (28)
W3		<u>35 (3.5)</u>	<u>33 (10)</u>	<u>28 (7)</u>	<u>62 (2)</u>	<u>34 (12)</u>	<u>79 (17)</u>
W4		<u>21 (3.5)</u>	<u>28 (7)</u>	5 (0)	6 (3)	25 (22)	<u>55 (3)</u>
DMG:							
W1		928 (1053)	417 (357)	564 (620)	650 (589)	401 (407)	509 (512)
W2		<u>678 (1028)</u>	<u>428 (617)</u>	<u>503 (718)</u>	<u>612 (584)</u>	<u>294 (514)</u>	410 (310)
W3		<u>661 (1000)</u>	<u>771 (642)</u>	<u>381 (657)</u>	<u>520 (851)</u>	<u>375 (586)</u>	435 (440)
W4		<u>786 (1100)</u>	<u>550 (657)</u>	<u>764 (778)</u>	<u>641 (696)</u>	<u>473 (610)</u>	<u>451 (681)</u>
Contact pigs		Yes to 100%	Yes to 75%	Not tested	Not tested	Not tested	Not tested
transmission							
% of pulmonary lesions		25	75	0	25	25	12
% of ganglionic lesions		17	33	67	25	50	12

\* ND: not determined,

\*\* hyperthermia when the temperature is greater than 40°C,

\*\*\* range of maximum temperatures recorded at the individual level,

\*\*\*\* the percentage corresponds to the number of temperature recordings greater than 40°C divided by the total number of temperature recordings in the week on all of the pigs.

In this test, there was no wasting, at the very most a retardation of the growth in the second, third or fourth week after infection. These data illustrate that certain breeding conditions probably favor the expression of the disease.

c) Tests No. 3 to No. 7: Reproduction of the experimental tests

5           The increase in the number of the experimental tests on pigs had the mastering and better characterization of the experimental model as an objective. All of the results are presented in Table 5.

10           Under the experimental conditions, PWD is thus characterized by a long incubation, of 8 to 14 days, true hyperthermia over 2 to 8 days, a decrease in food consumption and a retardation of the increase in weight on the second, third or fourth week post-infection. The lesional table associated with this clinical expression includes, in the main, ganglionic hypertrophy and lesions of pneumonia.

Conclusion

15           The perfection of this experimental model allows the direct etiological role of the PWD circovirus in the disease to be indisputably demonstrated. In addition, this model is an indispensable tool for the understanding of pathogenic mechanisms and the study of future vaccine candidates.

20           EXAMPLE 5: Demonstration of the vaccine composition protective efficacy produced from nucleic fragments of PWD circovirus sequence

1) Animals used for the study

25           Piglets having the PWD disease, reproduced under experimental conditions described in paragraph c) of Example 4, were used in a protocol for evaluating the vaccine composition efficacy, comprising nucleic fragments of PWD circovirus sequence.

2) Tested vaccine composition and vaccination protocol

a) Components used for the study

The plasmids were obtained from the pcDNA3 plasmid of INVITROGENE  
- pcDNA3ORF- plasmids

These plasmids are plasmids which do not carry a PWD circovirus nucleic acid insert and are used as a negative control plasmid.

- pcDNA3ORF1+ plasmid and pcDNA3ORF2+ plasmid

5 The pcDNA3ORF1+ and pcDNA3ORF2+ plasmids are plasmids which carry a nucleic acid insert of the sequence of the PWD circovirus of TYPE B, respectively an insert comprising the nucleic acid fragment SEQ ID No. 23 (ORF'1) coding for the Rep protein of sequence SEQ ID No. 24 and an insert comprising the nucleic acid fragment SEQ ID No. 25 (ORF'2) coding for the protein of sequence SEQ ID No. 26, probably corresponding to the capsid protein, these nucleic  
10 constructs comprising the ATG initiation codon of the coding sequence of the corresponding protein.

- GMCSF+ plasmid

GM-CSF (granulocyte/macrophage colony stimulating factor) is a cytokine which occurs in the development, the maturation and the activation of macrophages,  
15 granulocytes and dendritic cells which present an antigen. The beneficial contribution of the GM-CSF in vaccination is considered to be a cellular activation with, especially, the recruitment and the differentiation of cells which present an antigen.

This pcDNA3-GMCSF+ plasmid carries a nucleic acid insert coding for the granulocyte/macrophage colony stimulation factor, the GM-CSF protein.  
20

The gene coding for this GM-CSF protein was cloned and sequenced by Inumaru et al. (Immunol. Cell Biol., 1995, 73 (5), 474-476). The pcDNA3-GMCSF+ plasmid was obtained by Dr. B. Charley of INRA of Jouy-en-Josas (78, France).

25 - Recombinant baculoviruses

The so-called ORF- baculoviruses are viruses not carrying any insert comprising a nucleic acid fragment capable of expressing a PWD circovirus protein.

The so-called ORF1+ (BAC ORF1+) or ORF2+ (BAC ORF2+) baculoviruses are recombinant baculoviruses respectively carrying an insert

comprising a nucleic acid fragment SEQ ID No. 23 (ORF'1) and an insert comprising the nucleic acid fragment SEQ ID No. 25 (ORF'2).

- Adjuvant

5 The adjuvant supplied by the Seppic Company, a subsidiary of AIR LIQUIDE, is the adjuvant corresponding to the reference AIF SEPPIC.

b) Vaccination protocol

Weaned piglets aged 3 weeks are divided into four batches A, B, C and D each comprising 8 piglets.

10 Batches A, B and C, aged 3 weeks, each receive a first injection (injection M1) of 1 ml containing 200 micrograms of plasmids (naked DNA) in PBS, pH: 7.2, by the intramuscular route for each of the plasmids mentioned below for each batch, then, at the age of 5 weeks, a second injection (injection M2) comprising these same plasmids. A third injection is carried out simultaneously on the other side of the neck. This third injection comprises 1 ml of a suspension containing  $5 \cdot 10^6$  cells  
15 infected by recombinant baculoviruses and 1 ml of AIF SEPPIC adjuvant.

Batch A (F1) (control batch):

- first injection

pcDNA3ORF1- plasmid, pcDNA3ORF2- plasmid and GMCSF+ plasmid.

- second and third injection (simultaneous)

20 pcDNA3ORF1- plasmid, pcDNA3ORF2- plasmid and GMCSF+ plasmid;

Cells transformed by baculoviruses not containing any nucleic acid insert coding for a PWD circovirus protein;

AIF SEPPIC adjuvant.

Batch B (F2) (control batch):

25 - first injection

pcDNA3ORF1- plasmid, pcDNA3ORF2- plasmid and GMCSF+ plasmid;

- second and third injection (simultaneous)

pcDNA3ORF1- plasmid, pcDNA3ORF2- plasmid and GMCSF+ plasmid;

30 Cells transformed by baculoviruses not containing any nucleic acid insert coding for a PWD circovirus protein;



AIF SEPPIC adjuvant.

Batch C (F3):

- first injection

5 pcDNA3ORF1+ plasmid, pcDNA3ORF2+ plasmid and GMCSF+ plasmid;

- second and third injection (simultaneous)

pcDNA3ORF1+ plasmid, pcDNA3ORF2+ plasmid and GMCSF+ plasmid;

10 Cells transformed by BAC ORF1+ and BAC ORF2+ recombinant baculoviruses capable of respectively expressing the Rep protein of sequence SEQ ID No. 24 and the protein of sequence SEQ ID No. 26 of the PWD circovirus of TYPE B.

Batch D (F4) (control batch): no injection

15 The batches of piglets B, C and D are infected (tested) at the age of 6 weeks although batch A is not subjected to the test.

3) Observation of the batches

- 20
- counting of coughing/sneezing: 15 minutes/batch/day;
  - consistency of fecal matter: every day;
  - regular recordings: weekly taking of blood, weighing;
  - weighing of food refuse: 3 times per week;
  - calculation of the daily mean gain in weight (dmg);

25 The daily mean gains were calculated for each of the batches over a period of 28 days following testing (cf. Figure 10), an intermediate calculation of the dmg was likewise carried out for each of the batches over the first and second periods of 14 days. The results obtained are reported below in Table 6.

Table 6: Daily mean gains

	F1	F2	F3	F4
d0-d14	411 g	450 g	511 g	461 g
d14-d28	623 g	362 g	601 g	443 g
d0-d28	554 g	406 g	556 g	452 g

- Measurement of hyperthermia

5 The measurement of hyperthermia, of greater than 41°C (cf. Figure 11) and greater than 40.2°C, was carried out for each of the batches over a total period of 28 days following testing. The results obtained, corresponding to the ratio expressed as a percentage between the number of recordings of heat of greater than 41°C (or greater than 40.2°C) and the total number of recordings of heat carried out on all of the pigs per one-week period are reported below in Tables 7 and 8, respectively for

10 the hyperthermia measurements of greater than 41°C and greater than 40.2°C.

Table 7: Hyperthermia > 41°C

	F1	F2	F3	F4
W1	4.1	0.	0.	0.
W2	10.7	16.	0.	8.9
W3	4.7	27.	0.	45.
W4	0.	0.	0.	7.5

Table 8: Hyperthermia > 40.2

	F1	F2	F3	F4
W1	29.1	10.41	29.1	20.8
W2	28.5	39.2	10.7	37.5
W3	14.3	68.7	25.0	81.2
W4	3.3	17.5	20.0	55

15

#### 4) Conclusion

The recordings carried out clearly show that the animals which received the three injections of a vaccine composition comprising nucleic acid fragments of PWD circovirus according to the invention and/or capable of expressing recombinant proteins of PWD circovirus, in particular of type B, did not exhibit hyperthermia (cf. Figure 10). These animals additionally did not experience a decline in their growth, the dmgs being comparable to those of uninfected control animals (cf. Figure 9). They did not exhibit any particular clinical sign.

These results demonstrate the efficacious protection of the piglets against infection with a PWD circovirus of the invention, the primary agent responsible for PWD or FPW, provided by a vaccine composition prepared from a nucleic acid fragment of the nucleic sequence of PWD circovirus according to the invention, in particular of type B, and/or from recombinant proteins encoded by these nucleic acid fragments.

These results in particular show that the proteins encoded by the ORF1 and ORF2 of PWD circovirus according to the invention are immunogenic proteins inducing an efficacious protective response for the prevention of infection by a PWD circovirus.

EXAMPLE 6: Serological diagnosis of PWD circovirus by immunodetermination using recombinant proteins or synthetic peptides of PWD circovirus

A - Serological diagnosis with recombinant proteins

The identification and the sequencing of porcine PWD circovirus allow recombinant proteins of PWD circovirus to be produced by the techniques of genetic recombination well known to the person skilled in the art.

By these techniques, recombinant proteins encoded, in particular, by the ORF'2 of the PWD circovirus, type B, were expressed by transformed Sf9 insect cells and then isolated.

These recombinant proteins encoded by the ORF'2 are extracted, after culture of the transformed Sf9 cells, by thermal cell lysis by means of 3 cycles of

freezing/thawing to  $-70^{\circ}\text{C}/+37^{\circ}\text{C}$ . Healthy Sf9 cells or nontransformed control Sf9 cells are also lyzed.

These two antigenic fractions originating from nontransformed control Sf9 cells and Sf9 cells expressing the ORF'2 are precipitated at  $4^{\circ}\text{C}$  by a 60% plus or minus 5% saturated ammonium sulfate solution. Determination of total proteins is carried out with the aid of the Biorad kit. 500 ng of control Sf9 proteins and of semipurified Sf9 proteins expressing the ORF'2, in solution in 0.05 M bicarbonate buffer pH 9.6, are passively adsorbed at the bottom of 3 different cupules of a Nunc Maxisorp microplate by incubation for one night at  $+4^{\circ}\text{C}$ .

The reactivity of pig sera with respect to each of these antigenic fractions is evaluated by an indirect ELISA reaction of which the experimental protocol is detailed below:

- Saturation step: 200  $\mu\text{l}$ /cupule of PBS1X/3% semi-skimmed milk, 1 h 30 incubation at  $37^{\circ}\text{C}$ .
- Washing: 200  $\mu\text{l}$ /cupule of PBS1X/Tween 20: 0.05%, 3 rapid washes.
- Serum incubation step: 100  $\mu\text{l}$ /cupule of serum diluted to 1/100 in PBS1X/semi-skimmed milk, 1%/Tween 20: 0.05%, 1 h incubation at  $37^{\circ}\text{C}$ .
- Washing: 200  $\mu\text{l}$ /cupule of PBS1X/Tween 20: 0.05%, 2 rapid washes followed by 2 washes of 5 min.
- Conjugate incubation step: 50  $\mu\text{l}$ /cupule of rabbit anti-pig conjugate diluted to 1/1000 in PBS1X/semi-skimmed milk, 1%/Tween 20: 0.05%, 1 h incubation at  $37^{\circ}\text{C}$ .
- Washing: 200  $\mu\text{l}$ /cupule of PBS1X/Tween 20: 0.05%, 2 rapid washes followed by 2 washes of 5 min.
- Visualization step: 100  $\mu\text{l}$ /cupule of OPD substrate/citrate buffer/ $\text{H}_2\text{O}_2$ , 15 min incubation at  $37^{\circ}\text{C}$ .
- Stopping of reaction: 50  $\mu\text{l}$ /cupule of 1 N  $\text{H}_2\text{SO}_4$ .
- Reading in a spectrophotometer at 490 nm.

### Results

The results obtained are shown below in Table 9.

Table 9

Antigens	Reactivity of Pig Serum not inoculated with Circovirus	Reactivity of Pig Serum inoculated with Circovirus
Purified Sf9 control	0.076	0.088
Sf9 expressing purified ORF'2	0.071	1.035

5 The results are expressed in optical density measured in a spectrophotometer at 490 nm during analysis by ELISA of the reactivity of pig sera which are or are not inoculated with the type B PWD circovirus according to the protocol indicated above.

#### B - Serological Diagnosis by Synthetic Peptide

10 The epitopic mapping of the proteins encoded, for example, by the nucleic sequences ORF1 and ORF2 of the two types of PWD circovirus (types A and B) additionally allowed immunogenic circoviral epitopes to be identified on the proteins encoded by the nucleic sequences ORF'1 and ORF'2 as well as the specific epitopes of the protein encoded by the nucleic sequence ORF'2 of the type B PWD circovirus. Four specific epitopes of the type B PWD circovirus and one epitope  
15 common to the two types of PWD circovirus situated on the protein encoded by the nucleic sequence ORF'2 were synthesized in peptide form. The equivalent peptides in the circovirus of type A were likewise synthesized. All these peptides were evaluated as diagnostic antigens within the context of carrying out a serological test.

#### Results

20 The results obtained are shown in Table 10 below.

Table 10: Results of the evaluation as a diagnostic antigen of synthetic peptides encoded by the nucleic sequences ORF2 and ORF'2 of PWD circovirus of type A and B.

		Infected pig serum reactivity				
		Circovirus B				
SEQ ID NO:	Peptide	Type	Position	AA sequence	SPF D0/D54	Conventional 1 D0/D42
29	121	B	71-85	VDMMRFNINDELPPG	+/-, +++	+/-, +++
55	177	B	70-84	NVNELRFNIGQFLPP	+/-, +	+/-, +
30	131	B	115-129	QGDRGVGSSAVILDD	+/-, +/-	+, +
56	188	A	114-127	TSNQRGVGSTVVIL	+/-, -	+/-, +/-
31	133	B	119-134	GVGSSAVILDDNVFTK	-, ++	+, +
57	189	A	118-132	RGVGSTVVILDANFV	+/-, -	+/-, +/-
58	146	B	171-185	FTIDYFQPNKRNQL	-, +/-	-, ++
59	202	A	170-184	DQTIDWFQPNKRNQ	+++	+, +
32	152	B	195-209	VDHVGLGTAFENSIY	-, ++	+, +
60	208	A	194-208	NVEHTGLGYALQNAT	-, -	-, -
						Circovirus B

+/-, +, +, +, +. Increasing intensities of the reactivities observed in Spot peptides on a nitrocellulose membrane. The porcine sera tested are from animals experimentally infected with the circovirus of type B within the animal houses of the CNEVA. Samples are taken from the animals before inoculation on d0 and 42 days or 54 days after inoculation, on d42, d54.

EXAMPLE 7: Characterization of the specific epitopes of the PWD circovirus of type B

5 The proteins encoded by the ORF2 of the porcine circoviruses of type A and B were chosen for this study. For each of the ORF2s (types A and B), 56 peptides of 15 amino acids which overlap every 4 amino acids were synthesized, thus covering the whole of the protein (cf. Table 11 below).

10 Table 11: Sequence of amino acids of the 56 peptides of 15 amino acids synthesized from the nucleic sequence ORF'2 (type B) and ORF2 (type A) of PWD circovirus with their corresponding spot number (cf. Figure 12)

Type B ORF'2			Type A ORF2		
	Spot No.	Sequence		Spot No.	Sequence
SEQ ID NO:61	107	HRPRSHLGQILRRRP	SEQ ID NO:84	163	TRPRSHLGNILRRRP
SEQ ID NO:62	108	SHLGQILRRRPWL VH	SEQ ID NO:85	164	SHLGNILRRRPYLVH
SEQ ID NO:63	109	QILRRRPWL VHPRHR	SEQ ID NO:86	165	NILRRRPYLVHPAFR
SEQ ID NO:64	110	RRPWL VHPRHRYRWR	SEQ ID NO:87	166	RRPYLVHPAFRNRYR
SEQ ID NO:65	111	LVHPRHRYRWRRKNG	SEQ ID NO:88	167	LVHPAFRNRYRWRRK
SEQ ID NO:66	112	RHRYRWRRKNGIFNT	SEQ ID NO:89	168	AFRNRYRWRRKTGIF
SEQ ID NO:67	113	RWRRKNGIFNTRL SR	SEQ ID NO:90	169	RYRWRRKTGIFNSRL
SEQ ID NO:68	114	KNGIFNTRL SRTFGY	SEQ ID NO:91	170	RRKTGIFNSRLSREF
SEQ ID NO:69	115	FNTRL SRTFGYTVKR	SEQ ID NO:92	171	GIFNSRLSREFVLT I
SEQ ID NO:70	116	LSRTFGYTVKR TTVR	SEQ ID NO:93	172	SRLSREFVLTIRGGH
SEQ ID NO:71	117	FGYTVKR TTVRTPSW	SEQ ID NO:94	173	REFVLTIRGGHSQPS
SEQ ID NO:72	118	VKR TTVRTPSWAVDM	SEQ ID NO:95	174	LTIRGGHSOPSWNVN
SEQ ID NO:73	119	TVRTPSWAVDMMRFN	SEQ ID NO:96	175	GGHSQPSWNVNELRF
SEQ ID NO:74	120	PSWAVDMMRFNINDF	SEQ ID NO:97	176	QPSWNVNELRFNIGO
SEQ ID NO:29	121	VDMMRFNINDFLPPG	SEQ ID NO:98	177	NVNELRFNIGQFLPP
SEQ ID NO:75	122	RFNINDFLPPGGGSN	SEQ ID NO:99	178	LRFNIGQFLPPSGGT
SEQ ID NO:76	123	NDFLPPGGGSNPRSV	SEQ ID NO:100	179	IGQFLPPSGGTNPLP
SEQ ID NO:77	124	PPGGGSNPRSVPF EY	SEQ ID NO:101	180	LPPSGGTNPLPLPFQ
SEQ ID NO:78	125	GSNPRSVPF EYYRIR	SEQ ID NO:102	181	GGTNPLPLPFQYYRI
SEQ ID NO:79	126	RSVPF EYYRIRKVKV	SEQ ID NO:103	182	PLPLPFQYYRIRKAK
SEQ ID NO:80	127	FEYYRIRKVKVEFWP	SEQ ID NO:104	183	PFQYYRIRKAKYEFY
SEQ ID NO:81	128	RIRKVKVEFWPCSPI	SEQ ID NO:105	184	YRIRKAKYEFYPRDP
SEQ ID NO:82	129	VKVEFWPCSPITQGD	SEQ ID NO:106	185	KAKYEFYPRDPITSN
SEQ ID NO:83	130	FWPCSPITQGDRGVG	SEQ ID NO:107	186	EFYPRDPITSNQRGV
SEQ ID NO:30	131	SPITQGDRGVGSSAV	SEQ ID NO:108	187	RDITSNQRGVGSTV
SEQ ID NO:31	132	QGDRGVGSSAVILDD	SEQ ID NO:109	188	TSNQRGVGSTVVILD
SEQ ID NO:110	133	GVGSSAVILDDNFVT	SEQ ID NO:136	189	RGVGSTVVILDANFV
SEQ ID NO:111	134	SAVILDDNFVTKATA	SEQ ID NO:137	190	STVVILDANFVTPST
SEQ ID NO:112	135	LDDNFVTKATALTYD	SEQ ID NO:138	191	ILDANFVTPSTNLAY
SEQ ID NO:113	136	FVTKATALTYDPYVN	SEQ ID NO:139	192	NFVTPSTNLAYDPYI
SEQ ID NO:114	137	ATALTYDPYVNYSSR	SEQ ID NO:140	193	PSTNLAYDPYINYSS
SEQ ID NO:115	138	TYDPYVNYSSRIITIT	SEQ ID NO:141	194	LAYDPYINYSSRHTI
SEQ ID NO:116	139	YVNYSSRHTITQPFS	SEQ ID NO:142	195	PYINYSSRHTIRQPF



Type B ORF2			Type A ORF2		
	Spot No.	Sequence		Spot No.	Sequence
SEQ ID NO:117	140	SSRHTITQPFSYHSR	SEQ ID NO:143	196	YSSRIITIRQPFTYHS
SEQ ID NO:118	141	TITQPFSYHSRYFTP	SEQ ID NO:144	197	HTIRQPFTYHSRYFT
SEQ ID NO:119	142	PFSYHSRYFTP KPVL	SEQ ID NO:145	198	QPFTYHSRYFTP KPE
SEQ ID NO:120	143	HSRYFTP KPVL DFTI	SEQ ID NO:146	199	YHSRYFTP KPELDQT
SEQ ID NO:121	144	FTP KPVL DFTIDYYFQ	SEQ ID NO:147	200	YFTP KPELDQTIDWF
SEQ ID NO:122	145	PVL DFTIDYFQPNNK	SEQ ID NO:148	201	KPELDQTIDWFQPNN
SEQ ID NO:123	146	FTIDYFQPNNKRNQL	SEQ ID NO:149	202	DQTIDWFQPNNKRNQ
SEQ ID NO:124	147	YFQPNNKRNQLWLRL	SEQ ID NO:150	203	DWFQPNNKRNQLWLH
SEQ ID NO:125	148	NNKRNQLWLRLQTAG	SEQ ID NO:151	204	PNNKRNQLWLHLNTH
SEQ ID NO:126	149	NQLWLRLQTAGNVDH	SEQ ID NO:152	205	RNQLWLHLNTHTNVE
SEQ ID NO:127	150	LRLQTAGNVDHVGLG	SEQ ID NO:153	206	WLHLNTHTNVEHTGL
SEQ ID NO:128	151	TAGNVDHVGLGTAFE	SEQ ID NO:154	207	NHTNVEHTGLGYAL
SEQ ID NO:32	152	VDHVGLGTAFENSIY	SEQ ID NO:155	208	NVEHTGLGYALQNAT
SEQ ID NO:129	153	GLGTAFENSIYDQEY	SEQ ID NO:156	209	TGLGYALQNATTAQN
SEQ ID NO:130	154	AFENSIYDQEYNIRV	SEQ ID NO:157	210	YALQNATTAQNYVVR
SEQ ID NO:131	155	SIYDQEYNIRVTMYV	SEQ ID NO:158	211	NATTAQNYVVRLTIY
SEQ ID NO:132	156	QEYNIRVTMYVQFRE	SEQ ID NO:159	212	AQNYVVRLTIYVQFR
SEQ ID NO:133	157	IRVTMYVQFRENFNK	SEQ ID NO:160	213	VVRLTIYVQREFIL
SEQ ID NO:134	158	MYVQFRENFNKDPPL	SEQ ID NO:161	214	TIYVQFRENFILKDPL
SEQ ID NO:135	159	VQFRENFNKDPPLNP	SEQ ID NO:162	215	YVQFRENFILKDPLNE

These peptides were synthesized according to the "spot" method which consists in simultaneous synthesis of a large number of peptides on a cellulose solid support, each site of synthesis of a peptide constituting a spot (Synt:em, NIMES).

5 This method involves orientation of the peptides on the plate, these being fixed covalently by the carboxy-terminal end. A spot represents approximately 50 nmol of peptide.

The reference of the spots and corresponding peptide sequences is given in Table 11.

10 These membranes were used for immunoreactivity tests with respect to serum of SPF pigs which were or were not infected experimentally with the type B PWD circoviral strain as well as with respect to sera of infected pigs from conventional farms (conventional farms 1 or 2). This study allowed specific immunoreactive peptides of the circovirus of type B corresponding to the spots No.

15 121, No. 132, No. 133 and No. 152 (respectively of amino acid sequences SEQ ID No. 29, SEQ ID No. 30, SEQ ID No. 31 and SEQ ID No. 32) to be demonstrated. An illustration is shown in Figure 12 where the membranes are visualized with an

infected pig serum coming from a conventional farm. Nonspecific immunoreactive peptides of type [lacuna] were likewise demonstrated, among which we shall keep the peptide No. 146 SEQ ID No. 123 which is strongly immunogenic.

5 A comparison between the peptide sequences of circoviruses of type A and B (Figure 13) indicates a divergence ranging from 20 to 60% for the specific immunoreactive peptides of the type B, and a weaker divergence (13%) between the nonspecific peptides.

10 EXAMPLE 8: Protection of Swine From Post-Weaning Multisystemic Wasting Syndrome (PMWS) Conferred by Procine Circovirus TypeB (PCV-B) ORF'2 Protein

15 The ORF'1-encoded protein (REP) and ORF'2-encoded putative capsid protein of PCV-B were expressed, either in insect cells by recombinant baculovirus vectors, or in mammalian cell lines by transfection with plasmidic expression vectors. These two circovirus-derived proteins were detectable in both expression system. As evaluated by weight gains, hyperthermia and absence of lesions following challenge, the pigs were protected against a virulent circovirus challenge after one first DNA immunization with plasmids directing ORF'2 protein and GM-CSF expression and a second injection, 15 days later, with the same plasmid preparation plus the ORF'2 recombinant protein. A lower level of protection was observed when the pigs were vaccinated with ORF'1 protein, as opposed to pigs vaccinated with ORF'2 protein.

A. Development of an experimental model of PMWS in swine:

25 Eight 3 week-old SPF pigs were inoculated intratracheally (5 ml) and intramuscularly (1 ml).

B. Production and control of PCV-B plasmids:

PCV-B ORF'1 and ORF'2 genes , isolated from PCV-B challenge strain, have been cloned into vector plasmid pcDNA3.1.

All constructs have been validated through a partial sequencing of the PCV-B genes in the final plasmids and expression control by immunoperoxidase on PK15 cells respectively transfected with each plasmid , using swine polyclonal antibodies. Plasmid encoding GM-CSF has been co-administred.

5 C. Construction of recombinant baculoviruses:

ORF'1 and ORF'2 proteins were expressed under polyhedrin promoter control. Recombinant proteins were detected by western-blot using swine polyclonal antibodies.

D. Vaccination and challenge:

10 Four groups of 7 pigs were vaccinated intramuscularly at day 0 (Do), two weeks later, they received the same plasmid preparation plus the recombinant baculovirus.

E. Monitoring:

15 All groups of pigs were housed in isolated experimental units with air filtration and low air pressure. Clinical observations and rectal temperatures were recorded every day. The pigs were weighed weekly.

F. Conclusions

20 Expression of PCV-B ORF'2 or PCV-B ORF'1 in swine resulted in a significantly enhanced level of protection as evaluated by weight evolution and body temperature evolution following challenge with PCV-B circovirus. These results are summarized in Figures 14 and 15.

25 The invention described herein may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The specific embodiments previously described are therefore to be considered as illustrative of, and not limiting, the scope of the invention. Additionally, the disclosure of all publications and patent applications cited above and below, including International Patent Application No. PCT/FR98/02634, filed December 4, 1998, and published as

International Publication No. WO 99/29871 on June 17, 1999, are expressly incorporated herein by reference in their entireties to the same extent as if each were incorporated by reference individually.

**Bibliographic references**

- Allan, G.M. et al., 1995, Vet. Microbiol., 44: 49-64.
- Barany, F., 1911, PNAS. USA, 88: 189-193.
- Boulton, L.H. et al., 1997, J. Gen. Virol., 78 (Pt 6), 1265-1270.
- Buckholz, R.G., 1993, Yeast systems for the expression of heterologous gene products. Curr. Op. Biotechnology 4: 538-542.
- Burg, J.L. et al., 1996, Mol. and Cell. Probes, 10: 257-271.
- Chu, B.C.F. et al., 1986, NAR, 14: 5591-5603.
- Chu, P.W.G. et al., 1993, Virus Research, 27: 161-171.
- Clark, E.G., 1997, American Association of Swine Practitioners, 499-501.
- Daft, B. et al., 1996, American Association of Veterinary Laboratory Diagnosticians, 32.
- Derse, D. et al., 1995, J. Virol., 69(3): 1907-1912.
- Duck, P. et al., 1990, Biotechniques, 9: 142-147.
- Dulac, G.C. et al., 1989, Can. J. Vet. Res., 53: 431-433.
- Edwards, C.P., and Aruffo, A., 1993, Current applications of COS cell based transient expression systems. Curr. Op. Biotechnology 4: 558-563.
- Edwards, S. et al., 1994, Vet. Rec., 134: 680-681.
- Erlich, H.A., 1989, In PCR Technology. Principles and Applications for DNA Amplification. New York: Stockton Press.
- Felgner, et al., 1987, Proc. Natl. Acad. Sci., 84: 7413.
- Fontes, E.P.B. et al., 1994, J. Biol. Chem., Vol. 269, No. 11: 8459-8465.
- Fraley et al., 1980, J. Biol. Chem., 255: 10431.
- Guateli, J.C. et al., 1990, PNAS. USA, 87: 1874-1878.
- Hackland, A.F. et al., 1994, Arch. Virol., 139: 1-22.
- Hanson, S.F. et al., 1995, Virology, 211: 1-9.
- Harding, J.C., 1997, American Association of Swine Practitioners, 503.
- Harding, R.M. et al., 1993, Journal of General Virology, 74: 323-328.

- Harding, J.C. and Clark, E.G., 1997, Swine Health and Production, Vol. 5, No. 5: 201-203.
- Heyraud-Nitschke, F. et al., 1995, Nucleic Acids Research, Vol. 23, No. 6.
- Horner, G.W., 1991, Surveillance 18(5): 23.
- Houben-Weyl, 1974, in Methode der Organischen Chemie, E. Wunsch Ed., Volume 15-I and 15-II, Thieme, Stuttgart.
- Huygen, K. et al., 1996, Nature Medicine, 2(8): 893-898.
- Innis, M.A. et al., 1990, in PCR Protocols. A guide to Methods and Applications, San Diego, Academic Press.
- Kaneda, et al., 1989, Science, 243: 375.
- Kievitis, T. et al., 1991, J. Virol. Methods, 35: 273-286.
- Kohler, G. et al., 1975, Nature, 256(5517): 495-497.
- Kwoh, D.Y. et al., 1989, PNAS. USA, 86: 1173-1177.
- Ladany, S. et al., 1989, J. Clin. Microbiol. 27: 2778-2783.
- Lazarowitz, S.G. et al., 1989, The EMBO Journal, Vol. 8 No. 4: 1023-1032.
- Luckow, V.A., 1993, Baculovirus systems for the expression of human gene products. Curr. Op. Biotechnology 4: 564-572.
- Mankertz, A. et al., 1997, J. Virol., 71: 2562-2566.
- Matthews, J.A. et al., 1988, Anal. Biochem., 169: 1-25.
- McNeilly, F. et al., 1996, Vet. Immunol. Immunopathol., 49: 295-306.
- Meehan, B.M. et al., 1997, J. Gen. Virol. 78: 221-227.
- Merrifield, R.D., 1966, J. Am. Chem. Soc., 88(21): 5051-5052.
- Midoux, 1993, Nucleic Acids Research, 21: 871-878.
- Miele, E.A. et al., 1983, J. Mol. Biol., 171: 281-295.
- Murphy, F.A. et al., 1995, Sixth Report of the International Committee on Taxonomy of Viruses. Springer-Verlag Wien New York.
- Nayar, G.P. et al., 1997, Can. Vet. J. 38(6): 385-386.
- Olins, P.O., and Lee, S.C., 1993, Recent advances in heterologous gene expression in E.coli. Curr. Op. Biotechnology 4: 520-525.

- Pagano et al., 1967, *J. Virol.*, 1: 891.
- Rolfs, A. et al., 1991, In *PCR Topics. Usage of Polymerase Chain reaction in Genetic and Infectious Disease*. Berlin: Springer-Verlag.
- Sambrook, J. et al., 1989, In *Molecular cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Sanchez-Pescador, R., 1988, *J. Clin. Microbiol.*, 26(10): 1934-1938.
- Segev D., 1992, in "Non-radioactive Labeling and Detection of Biomolecules". Kessler C. Springer Verlag, Berlin, New-York: 197-205.
- Shiver, J.W., 1995, in *Vaccines 1995*, eds Chanock, R.M. Brown, F. Ginsberg, H.S. & Norrby, E., pp. 95-98, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Tascon, R.E. et al., 1996, *Nature Medicine*, 2(8): 888-892.
- Tischer, I. et al., 1982, *Nature*, 295: 64-66.
- Tischer, I. et al., 1986, *Arch. Virol.*, 91: 271-276.
- Tischer, I. et al., 1988, *Zentralbl Bakteriol Mikrobiol Hyg [A]* 270: 280-287.
- Tischer, I. et al., 1995, *Arch. Virol.*, 140: 737-743.
- Urdea, M.S., 1988, *Nucleic Acids Research*, II: 4937-4957.
- Walker, G.T. et al., 1992, *NAR* 20: 1691-1696.
- Walker, G.T. et al., 1992, *PNAS. USA*, 89: 392-396.
- White, B.A. et al., 1997, *Methods in Molecular Biology*, 67, Humana Press, Towota.
- Zhao, T.M. et al., 1996, *Proc. Natl. Acad. Sci., USA* 93(13): 6653-6648.



We Claim:

1. A vaccine comprising a nucleotide sequence of the genome of Porcine circovirus type B, or a homologue or fragment thereof, and an acceptable pharmaceutical or veterinary vehicle.

2. A vaccine according to claim 1, wherein the nucleotide sequence is selected from SEQ ID No. 15 or SEQ ID No. 19.

3. A vaccine according to claim 1, wherein the homologue has at least 80% sequence identity to SEQ ID No. 15 or SEQ ID No. 19.

4. A vaccine according to claim 1, wherein the nucleotide sequence is selected from SEQ ID No. 23 or SEQ ID No. 25, or a homologue or fragment thereof.

5. A vaccine according to claim 4, wherein the homologue has at least 80% sequence identity to SEQ ID No. 23 or SEQ ID No. 25.

6. A vaccine according to claim 4, wherein the nucleotide sequence is SEQ ID No. 25.

7. A vaccine comprising a polypeptide encoded by a nucleotide sequence of the genome of PCVB, or a homologue or fragment thereof, and an acceptable pharmaceutical or veterinary vehicle.

8. A vaccine according to claim 7, wherein the homologue has at least 80% sequence identity to SEQ ID No. 15 or SEQ ID No. 19.

9. A vaccine according to claim 7, wherein the nucleotide sequence is selected from SEQ ID No. 23 or SEQ ID No. 25, or a homologue or fragment thereof.

10. A vaccine according to claim 9, wherein the homologue has at least 80% sequence identity to SEQ ID No. 23 or SEQ ID No. 25.

11. A vaccine according to claim 9, wherein the nucleotide sequence is SEQ ID No. 25.

12. A vaccine according to claim 7, wherein the polypeptide has the amino acid sequence of SEQ ID No. 24 or SEQ ID No. 26.

13. A vaccine according to claim 12, wherein the polypeptide has the amino acid sequence of SEQ ID No. 26.

14. A vaccine according to claim 7, wherein the homologue has at least 80% sequence identity to SEQ ID No. 24 or SEQ ID No. 26.

15. A vaccine according to claim 14, wherein the homologue has at least 80% sequence identity to SEQ ID No. 26.

16. A vaccine according to claim 7, wherein the polypeptide has the amino acid sequence of SEQ ID No. 29, SEQ ID No. 30, SEQ ID No. 31, or SEQ ID No. 32.

17. A vaccine comprising a vector and an acceptable pharmaceutical or veterinary vehicle, the vector comprising a nucleotide sequence of the genome of Porcine circovirus type B, or a homologue or fragment thereof.

18. A vaccine according to claim 17, further comprising a gene coding for an expression product capable of inhibiting or retarding the establishment or development of a genetic or acquired disease.

19. A vaccine comprising a cell and an acceptable pharmaceutical or veterinary vehicle, wherein the cell is transformed with a nucleotide sequence of the genome of Porcine circovirus type B, or a homologue or fragment thereof.

20. A vaccine according to claim 1, further comprising an adjuvant.

21. A vaccine comprising a pharmaceutically acceptable vehicle and a single polypeptide, wherein the single polypeptide consists of SEQ ID No. 26.

22. A method of immunizing a mammal against piglet weight loss disease comprising administering to a mammal an effective amount of the vaccine of any one of claims 1-21.

**Abstract of the Invention**

The genome sequences and the nucleotide sequences coding for the PWD circovirus polypeptides, such as the circovirus structural and non-structural polypeptides, vectors including the sequences, and cells and animals transformed by the vectors are provided. Methods for detecting the nucleic acids or polypeptides, and kits for diagnosing infection by a PWD circovirus, also are provided. Method for selecting compounds capable of modulating the viral infection are further provided. Pharmaceutical, including vaccines, compositions for preventing and/or treating viral infections caused by PWD circovirus and the use of vectors for preventing and/or treating diseases also are provided.